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MICROBIAL CELL WALLS

M. R. J. SALTON

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1956 H. A. Barker, *Bacterial Fermentations*

1957 E. P. Abraham, *Biochemistry of Some Peptide and Steroid Antibiotics*

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By M. R. J. SALTON

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PREFACE

Science, like art, music, and literature, is susceptible to fashions, and it has been my good fortune to be actively engaged in a field of research that has attracted many investigators in the last ten years. This has added much personal stimulus to the fascination of scientific research, and it is always a great pleasure to talk "shop" with an ever-growing circle of microbiologists and biochemists. An invitation to present three lectures at the distinguished Institute of Microbiology at Rutgers was an extremely happy event for me, as it enabled me to meet and talk with both old and new friends and to lecture on a topic I especially enjoy. For this two-fold pleasure I should like to express my warmest appreciation to CIBA Pharmaceutical Products Inc., whose generous support made these Lectures in Microbial Biochemistry possible.

The structure of the microbial cell has intrigued most microbiologists, and what has been particularly fascinating has been the discovery that their biochemical apparatus and

structural and functional elements are so neatly packaged into cells of such small dimensions. Because many of the anatomical parts of microbial cells were beyond the limits of resolution of the light microscope, little detailed knowledge of microbial structure could emerge until the introduction of electron microscopy. It was this coincidence of the development of electron microscopy with the accumulated wealth of biochemical information that paved the way for the investigators of the major structural components of microbial cells. This book, based on the lectures delivered at Rutgers, illustrates the successful application of the techniques of biophysics, chemistry, and biochemistry to one facet of microbial anatomy. The development of the studies on microbial walls has been a rapid one and has occurred in a number of laboratories. Thus we have already reached the stage where we can but survey the general field in three lectures. The material in this book, therefore, does not represent a complete record of investigations on microbial walls. It has been selected with the hope that it will give an orientation to the newcomer or interested reader and a more detailed record on several aspects of wall chemistry for the initiated investigator requiring a summary. In a field advancing with some rapidity it is inevitable that important papers will have appeared in the interim between the lectures and this published account, and it is the constant nightmare of all authors and reviewers that their works will be out of date by the time they are printed. This does not, I hope, negate the usefulness of a summary of events leading to the latest exciting addition to the study of microbial cell walls.

For my own small part in the development of this field of endeavor I owe much to the broad introduction to microbiology I received in Australia and the many years of interest, stimulation, and encouragement I enjoyed as a visitor

and later as a member of Professor E. F. Gale's unit in the Department of Biochemistry at Cambridge. For the preparation of electron micrographs used in the lectures and this book I am most grateful to Dr. J. A. Chapman of the Rheumatism Research Department, University of Manchester, Professor A. L. Houwink of the Technical Physics Department, Delft, Professor E. Kellenberger of the Laboratoire de Biophysique, Geneva, Dr. V. Mohr of the Department of Biochemistry, The Technical University of Norway, Professor R. G. E. Murray, Department of Bacteriology, London, Ontario, Dr. D. H. Northcote, Department of Biochemistry, Cambridge, and Professor R. C. Williams, University of California, Berkeley. I should also like to thank Dr. M. Ikawa and Professor E. E. Snell for their kind permission to quote their results prior to publication. It is a great pleasure to thank the members of the Institute of Microbiology at Rutgers for their hospitality during the presentation of these lectures.

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CHAPTER

1

ISOLATION AND GENERAL PROPERTIES OF MICROBIAL CELL WALLS

Microbial anatomy, that specialized branch of the study of the structure of microorganisms, has emerged in the last ten to twenty years and has greatly attracted the attention of the biochemist and the biophysicist. There are very good reasons for distinguishing between the cytologists of former periods and the modern microbial anatomists, for the latter now have to attempt to explain their observations in terms of the biochemical functions of the cell and the molecular structures of cellular subunits.

Our interest in microbial cell structure has, of course, a long history and really stems from Antonie van Leeuwenhoek's observations on the shapes and forms of various microorganisms. Just as Leeuwenhoek's microscope revealed a new and exciting world of small "animalcules," so in our day the electron microscope with all its associated techniques has taken us inside the cell itself and revealed many fascinating details of the macromolecular complexity of living organisms. Thus in the last two decades a great

deal has been learned about the structure, functions, and chemistry of the principal morphological entities (flagella, capsules, walls, membranes, and nuclei) and various subcellular particles and organelles of microorganisms.[1,2]

That most bacteria, yeasts, fungi, and algae are surrounded by a rigid wall was apparent to the early cytologists. Indeed, it would seem that Leeuwenhoek was sufficiently perspicacious to realize that his "animalcules" were bounded by some sort of structure. From his letter to the Royal Society (Dobell [3]) it is evident that he looked and expected to resolve what it was that "held them together." Little time was lost between the introduction of methods for growing microorganisms in pure culture and the first attempt to discover the chemical composition of a microbial cell wall. Vincenzi,[4] as long ago as 1887, was the first to investigate what he believed to be the wall of *Bacillus subtilis.*

Most of the early studies of cell-wall composition were based on analysis of material that resisted various solvents and extraction procedures designed to remove cellular components. We now know, of course, that the carbohydrate chemist's addiction to extracting tissues with alkali to obtain wall polysaccharides removed other constituents and really left only part of the "native" cell wall. Methods used for the isolation of chitin from higher organisms have been applied to microorganisms, and X-ray data, together with chemical analysis, have substantiated the presence of a chitin-like polymer in the walls of some fungi.[5,6] Thus Blank [5] found that the "chitin" fraction of a number of dermatophytes gave X-ray results and nitrogen values similar, if not identical, to those expected for pure chitin.

It is now generally conceded that the polymers isolated as "wall" or mycelial residues by extraction procedures used in the earlier studies do not represent the entire chemi-

cal structure of the wall as it occurs in the intact cell. Consequently, more refined and less drastic methods for isolating walls have been evolved, and mechanical disintegration of cells and tissues has become the universal starting point.

Isolation of Cell Walls

The isolation of microbial structures as homogeneous morphological entities has resulted from the application of the methods of biochemistry, biophysics, and electron microscopy. Weibull [7] was one of the first to use a combination of such methods for the isolation of a bacterial structure when he separated and characterized flagella from *Proteus vulgaris*. Although mechanical methods have been available for the disintegration of microorganisms for some time, they were not applied to the problem of isolating wall structures until Mudd, Polevitsky, Anderson, and Chambers [8] showed by electron microscopy that sonic disintegration of bacteria left a resistant wall. Dawson [9] later demonstrated the complete separation of cytoplasm from the wall of *Staphylococcus aureus* by disintegrating the cells with glass beads. It thus became apparent to several of us (Mitchell and Moyle,[10] Salton and Horne,[11] Salton [12]) that such procedures could be used in conjunction with differential centrifugation to obtain homogeneous preparations which could be submitted to the techniques of analytical chemistry for the elucidation of their nature.

The methods for isolating microbial cell walls follow well-known recipes, and as we are all familiar with what good and bad cooks can do with recipes we need not discuss the isolation procedures in any detail. Cells may be disintegrated and deprived of their cytoplasm by one of the following three methods:

1. Mechanical disintegration (disruption by violent agitation with beads,[9,10,11] sonic and ultrasonic disintegra-

tion,[13,14] decompression rupture,[15] pressure cell disintegrator [16])

2. Osmotic lysis
3. Autolysis [17]

Of the three methods, mechanical disintegration is preferable, and all of the major methods listed under (1) have been used successfully in wall isolation. The method of choice will depend on the particular organism, but it may be worth emphasizing that of the mechanical procedures tried disintegration by sound and supersound can lead to a greater breakdown of the wall structure than that encountered with the other methods.[18] Even the robust walls from *Staphylococcus aureus* can be rendered nonsedimentable by exposure in the 10-kc Raytheon for 30 to 60 minutes.[19] Marr and Cota-Robles [20] have also pointed out that concomitant with the disruption and release of ribosomes and intracellular particles from *Azotobacter vinelandii* there is a disintegration of the "envelope" structure. These effects of sonic disintegration of wall, or envelope, may account for the rather low yields of walls encountered by some investigators.

Disintegration is generally performed under conditions that minimize enzymic modification of the walls, and the methods devised by Shockman, Kolb, and Toennies [21] and Ribi, Perrine, List, Brown, and Goode [16] have great advantages in that the temperature can be controlled accurately during disruption. Many organisms contain enzymes capable of completely digesting their own cell walls. Strange and Dark [22] had difficulty in obtaining wall preparations of *Bacillus* spp. free of cell-wall degrading enzymes. Because of the risk of degrading the wall enzymically, lytic and autolytic methods of cell disintegration are not recommended. On the other hand, various enzymes have been

used with considerable advantage in the removal of cytoplasmic materials from crude cell-wall fractions. Thus ribonuclease, trypsin, and lipase can be used without destroying the rigidity of or apparently degrading the wall structure.

Owing to the small dimensions of microbial structures, the only satisfactory method of establishing their morphological homogeneity has been by electron microscopic examination. Cell walls can thus be differentiated from other structures such as flagella, fimbriae, ribosomes, and intracellular particles.

Electron Microscopy of Isolated Cell Walls

Microbial walls isolated by the foregoing procedures generally retain the shape and outline of the organism from which they had been derived. This fact, together with the morphological changes accompanying enzymic removal of walls with protoplast formation (Weibull [23]), makes it certain that it is the wall that confers the shape on a particular organism. Walls of rod-shaped organisms are typically cylindrical in shape on examination in the electron microscope and those of *Streptococcus faecalis* are ellipsoidal.[11]

Some of the first microbial walls isolated by mechanical methods showed no evidence of fine structure. The wall of baker's yeast isolated by Northcote and Horne [24] appeared as a thick amorphous structure on examination in the electron microscope. However, by treatment with alkali and acid successively, Houwink and Kreger [25] removed some of the matrix from the walls of *Candida tropicalis* and showed a microfibrillar structure in the walls of this yeast (Fig. 1). By using more selective methods of extracting wall compounds, Nickerson and his colleagues [26,27] were

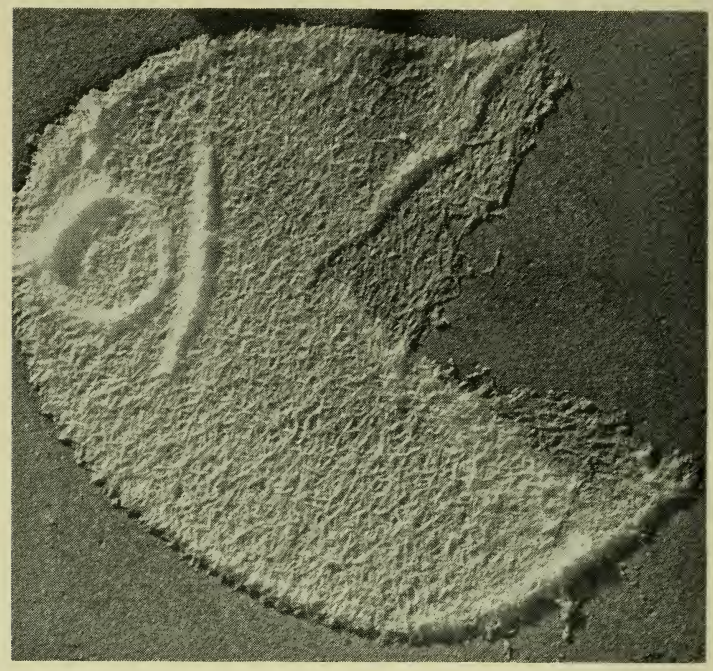
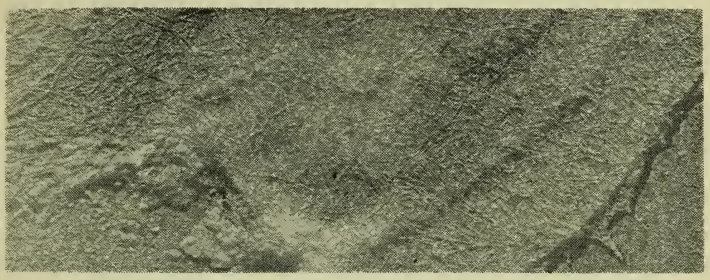


Fig. 1. Microfibrillar structure in the wall of *Candida tropicalis* (×18,500). By courtesy of Drs. Houwink and Kreger (Ref. 25).

able to show that the glucan component of baker's yeast wall possessed the fibrillar structure. The microfibrils in the yeast wall (Fig. 1) are arranged at roughly 90° to one another. However, around the bud scars the fibers are oriented differently, and Falcone and Nickerson [28] have proposed an explanation for the fiber orientation, based on a local explosion or "blow-out" of the wall during cellular division. Northcote, Goulding, and Horne [29] have also shown that by degradation of the isolated wall of *Chlorella*

pyrenoidosa with dilute solutions of sodium hydroxide a microfibrillar layer is revealed, and again the fibers lie at approximately 90° to one another (Fig. 2).

The presence of microfibrils in fungal cell walls has been reported by several investigators (Frey-Wyssling and Mühlethaler,[30] Roelofsen,[31] Shatkin and Tatum [32]). Roelofsen [31] found that the fibrils on the outer and inner layers of the



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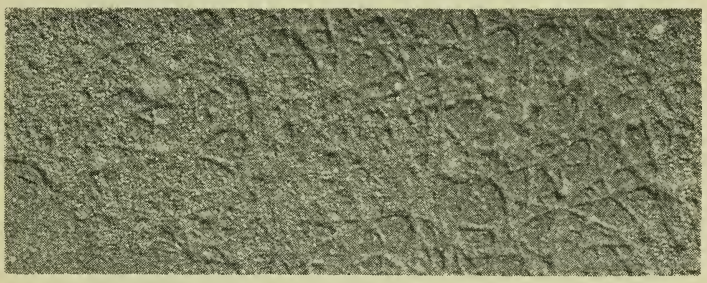


(*b*)

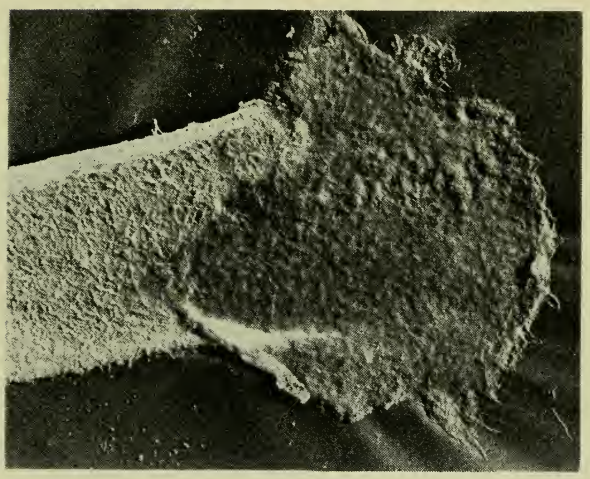
Fig. 2. Electron micrographs showing microfibrillar structure in the wall of *Chlorella pyrenoidosa*. (*a*) Walls treated with 0.5% NaOH for 30 minutes at room temperature (×21,000). (*b*) Walls treated with 3% NaOH for 30 minutes at room temperature (×39,000). By courtesy of Drs. Northcote, Goulding, and Horne (Ref. 29).

developing wall of *Phycomyces blakesleeanus* sporangiophores were 150 to 250 Å thick. The average fibril direction was too uncertain to suggest a spiral structure, but the inner layer showed a roughly transverse orientation. Thin sections of *Neurospora crassa* prepared by Shatkin and Tatum [32] showed a wall containing fine fibrils in a homogeneous matrix. The wall structure is much more readily shown in isolated mycelial fragments prepared by disintegration of *Neurospora crassa* by the methods used for bacterial cell-wall isolation. Figure 3*a* illustrates the appearance of an isolated mycelial wall with a rough outer texture and the more detailed microfibrillar structure of the wall in Fig. 3*b* (Chapman and Salton [33]).

No such fibrillar layer has been detected in the walls of bacteria, although the walls of *Bacillus megaterium* give a vague impression of being fibrous (Fig. 4). The walls of many Gram-positive bacteria, such as those of *Staphylococcus aureus* and *Streptococcus faecalis,* have a homogeneous appearance, and only thickened bands at what is presumed to be the site of new wall formation can be seen.

A type of fine structure differing from that observed in yeast walls and various algae [29,34] was first reported by Houwink [35] on examination of the wall of a large *Spirillum* species. The cell wall of this organism was a multilayered structure, with one layer composed of spherical macromolecules packed hexagonally. Such a macromolecular layer was also observed in the wall of *Spirillum serpens,* and Salton and Williams [36] found a similar type of fine structure in the wall of *Rhodospirillum rubrum.* This spherical macromolecular type of structure is apparently not uncommon, for Houwink [37] detected it also in the wall of *Halobacterium halobium.* Figure 5 illustrates the hexagonally packed macromolecular fine structure found in the wall of *Halobacterium halobium.*

(*a*)



(*b*)

Fig. 3. Electron micrographs of (*a*) cell walls of *Neurospora crassa* (×3,800); (*b*) microfibrillar structure in isolated cell wall of *Neurospora crassa* (×9,500). By courtesy of Drs. Chapman and Salton (Ref. 33).

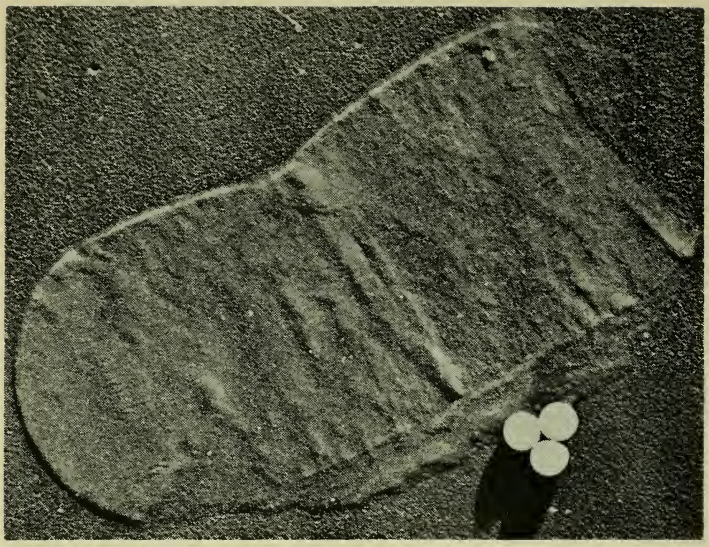


Fig. 4. Isolated cell wall of *Bacillus megaterium* (×21,000). By courtesy of Drs. Salton and Williams (Ref. 36).

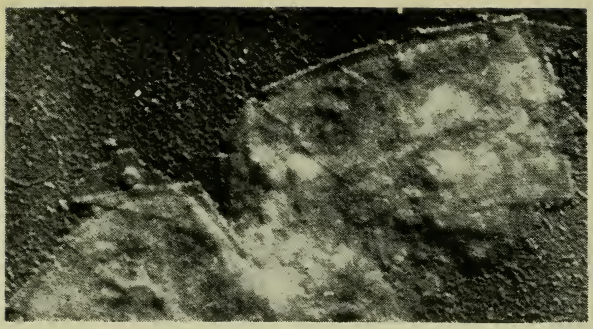
Fig. 5. Electron micrograph of *Halobacterium halobium* showing hexagonally packed macromolecules in the cell wall (×42,500). By courtesy of Drs. Houwink, Mohr, and Spit.

A different kind of microstructure in a bacterial wall was observed by Labaw and Mosley.[38] A rectangular array of macromolecules was found in the wall of an unidentified organism. More recently, yet another type of fine structure has been discovered in the wall of *Lampropedia hyalina* from observations made by Dr. J. A. Chapman (Rheumatism Research Department of the University of Manchester) and the author and independently by Dr. R. G. E. Murray. The outer layer of the wall of this organism possesses macromolecular subunits arranged to give the appearance of either a honeycomb network or an array of "knobs" spaced on a basal sheet—rather like a rubber mat. This type of structure gives rise to a "perforated edge" and lattice appearance as seen in isolated cell-wall fragments (Fig. 6).

In general, the macromolecules or their spacings in the fine-structured walls are of the order of 100 Å. The diameters of the large spherical macromolecules of the *Spirillum* sp. wall were 120 Å.[35]

Although bacteria such as *Escherichia coli* have shown no fine structure in the isolated walls when examined in the electron microscope by the usual methods, the thin sections prepared by Kellenberger and Ryter [39] have clearly established the multilayered nature of the wall. Thus, as shown in Fig. 7, it has been possible to differentiate a multilayered wall from the underlying membrane (presumably the protoplast membrane). As prepared for electron microscopy, the wall consisted of three layers, two of which were electron dense and one electron transparent, each of about 20 to 30 Å in thickness.

Thin sections of yeast [40] and *Chlorella pyrenoidosa* walls [29] have also confirmed the presence of several layers; they are probably double-layered structures. Thus, with microfibrillar layers in their walls, the yeasts, Chlorella, and some fungi closely resemble the wall structures found in



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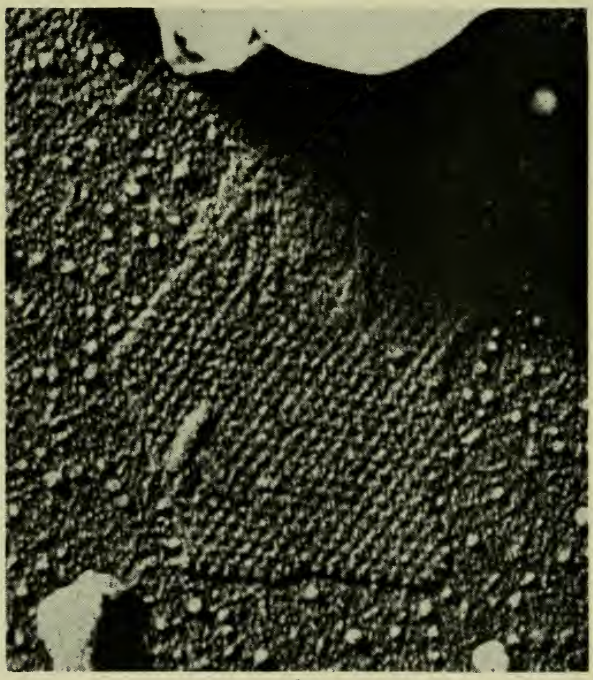
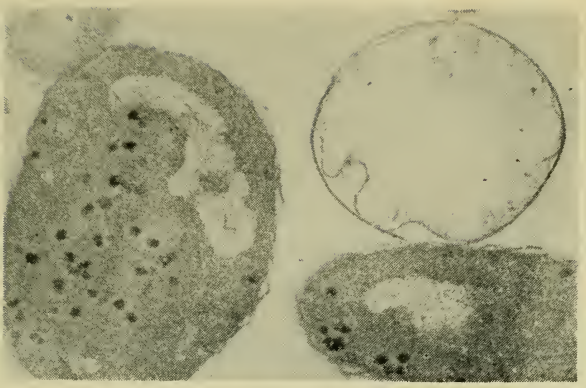


(*b*)

Fig. 6. (*a*) Isolated wall fraction from disintegrated *Lampropedia hyalina* (×37,000); (*b*) wall fragment showing typical lattice appearance (×102,000). By courtesy of Drs. Chapman and Salton, to be published.



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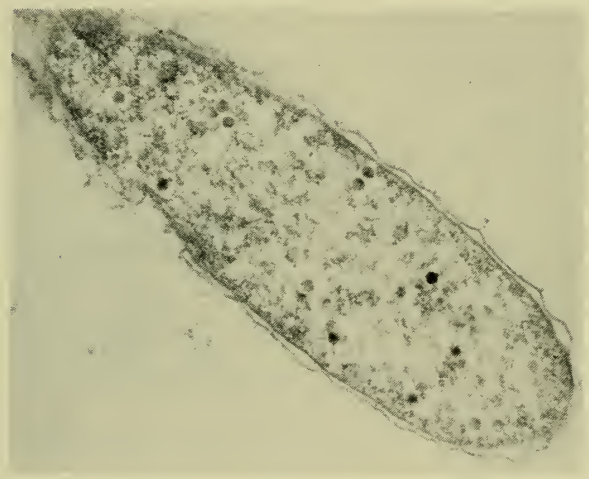

(*b*)

Fig. 7. Thin sections of *Escherichia coli* infected with bacteriophage. (*a*) Coccoid and lysed cells showing multilayered wall and underlying membrane (×32,500). By courtesy of Drs. Kellenberger and Ryter (Ref. 39). (*b*) Cell showing complete differentiation of wall, cytoplasmic membrane, and residual cytoplasm upon phage infection (×34,000). By courtesy of Drs. Kellenberger and Boy de la Tour, unpublished electron micrograph.

higher red, brown, and green algae and plants.[34] The types of structures detected in microbial walls are summarized in Table 1. Multilayered walls are encountered more fre-

TABLE 1

Physical Properties of Microbial Cell Walls Revealed by Electron Microscopy

	Type of Fine Structure
Algae	
Chlorella pyrenoidosa	Double-layered, microfibrillar polysaccharide (fibers at 90° to one another) + amorphous matrix
Fungi	
Phycomyces, *Neurospora crassa*	Microfibrillar components
Yeast	
Saccharomyces cerevisiae	Multilayered wall, microfibrillar layer (fibers 90° to one another); fibers oriented around bud scars
Bacteria	
Escherichia coli	Multilayered (2 electron dense: 1 electron transparent layers); macromolecules not visible in intact wall
Halobacterium, *Spirillum* sp., *Spirillum serpens*, *Rhodospirillum rubrum*	Multilayered structures with spherical macromolecules (80–120 Å diameter) visible; hexagonal packing
Lampropedia hyalina	Structure giving crystalline lattice appearance
Bacillus megaterium	Fibrous?
Staphylococcus aureus, *Streptococcus faecalis*	Amorphous structure—thickened bands at zone of wall formation

References 24 to 40.

quently in the Gram-negative group of bacteria, a difference in the level of complexity that could have been predicted from the early studies of chemical constitution.

General Physical Properties

The majority of microbial cell walls are fairly robust structures, and in many instances they must obviously be strong enough to withstand high pressures exerted upon them by those organisms capable of achieving a high concentration gradient across the wall-membrane (envelope). Mitchell and Moyle [41] found that the solute concentration in *Micrococcus lysodeikticus* and *Sarcina lutea* corresponded to an osmotic pressure of 20 atmospheres. The wall must therefore possess sufficient tensile strength to protect the cell against osmotic explosion. However, the walls of certain halophilic organisms are apparently not strong enough to prevent osmotic lysis when these bacteria encounter environments of low solute concentrations.[42]

The thickness of microbial walls has been reported by a number of investigators, either from thin sectioning of the cells or isolated walls or from direct measurement of the height of the shadows cast in specimens examined by electron microscopy. Some typical examples for various microbial walls are given in Table 2, together with data on the contribution of the wall to cell mass. There would seem to be some anomalies in the data for wall thickness, cell size, and weight contribution for the yeasts and Chlorella in particular, and the final assessment of the accuracy of these measurements will have to await further determinations.

It is evident that the wall accounts for a considerable proportion of the cell weight, the actual contribution depending on the phase of growth in the case of a bacterium such as *Streptococcus faecalis*.[43] Toennies and Shockman [44]

TABLE 2

Cell-Wall Thickness and Contribution to Cell Dry Weight

Organisms	Wall Thickness Å	Cell Walls as % Dry Weight Bacteria
Escherichia coli	100	15
Staphylococcus aureus	150–200	20
Streptococcus faecalis	200	27 (exponential phase) 38 (stationary phase)
Myxococcus xanthus	250	7–8
Chlorella pyrenoidosa	210	13.6
Yeast	1600	15 *

*Based on 90% recovery of various fractions from disintegrated yeast.

References 1, 24, 29, 39, 40, 43, 45.

have clearly shown that the nutritional status of the organism is of some importance in governing the amount of wall formed. When *Streptococcus faecalis* was grown under conditions of threonine depletion, the wall accounted for as much as 44% of the weight of the cell. No detailed investigations comparable to those of Shockman and Toennies appear to be available for microorganisms other than bacteria.

General Chemistry of Microbial Cell Walls

Before discussing in any detail the nature of the chemical constituents of microbial cell walls, let us first consider the major classes of substances encountered in these structures. It is generally agreed that nucleic acids are not major constituents of walls, although, as Barkulis and Jones [46] have

pointed out, small amounts of nucleic acid can be extracted from streptococcal (group A) wall preparations. Whether the nucleic acid or nucleic acid derivatives extractable from the wall are associated with it for "biochemical purposes" is not known. Contrary to the earlier views of Stacey,[47] it is now generally conceded that the nucleic acids are thus of minor importance in a consideration of the types of structural polymers in cell walls.

In addition to the nucleic acids, the cellular pigments also appear to be of intracellular origin, and there is no evidence of their being associated covalently with structural compounds encountered in nature. Although many pigmented organisms give wall fractions devoid of pigments, there is a number of instances in which these compounds persist in the wall fraction during isolation. Cell-wall fractions of several photosynthetic bacteria contain both carotenoids and photosynthetic pigments, although the latter are obviously much more abundant in the chromatophore fractions.[1,48] The presence of pigments in the wall fractions can, with some justification, be regarded with suspicion, and their presence may be an artifact of the isolation procedures. However, it may well be that in some organisms certain pigments are located in the wall of the intact cell. Isolated walls of the two blue-green algae, *Anacystis nidulans* and *Microcoleus vaginatus,* contained carotenoids, but the chlorophylls separated in a small particle fraction quite cleanly from the wall fractions (Salton, unpublished data).

In selecting results to illustrate the general features of the chemistry of cell walls, I have confined my choice largely to studies in which the wall structures have been isolated by mechanical disintegration and differential centrifugation. It became apparent during the search for this data that, apart from bacteria, little information is available for

other groups of microorganisms. Although there have been many studies of what has been assumed to be wall material after extraction of whole microorganisms with alkali, these studies have not been included in the present survey. The only comparative study of the old methods of isolating walls by alkaline digestion and the new methods by mechanical disintegration is that of Aronson and Machlis [49] for the walls of the fungus *Allomyces macrogynus*. Their results are presented in Table 3 and show a loss of wall constituents when isolation is performed by extraction with alkali.

Several typical analyses of the isolated walls of a yeast, a green alga, and a Gram-positive and a Gram-negative bacterium are summarized in Table 4. One conspicuous feature illustrated in Table 4 is the high amino sugar content of the wall of the Gram-positive organism in comparison to the other microorganisms. The major classes of sub-

TABLE 3

The Composition of Walls of *Allomyces macrogynus* Isolated by Alkaline Digestion and by Sonic Oscillation

Component	% of Dry Walls	
	Alkaline Digestion	Sonic Oscillation
Nitrogen	4.7	5.5
Acetyl	15.5	—
Moles N/moles acetyl	0.9	—
Protein	—	10
Chitin	68	58
Glucan	8	16
Ash	10	8

Reference 49.

TABLE 4

Comparative Cell-Wall Composition for Organisms from Several Microbial Groups

Chemical Constituent	% Dry Weight Cell Wall			
	Yeast (Baker's yeast)	Alga (*Chlorella pyrenoidosa*)	Bacteria (*Escherichia coli*)	(*Streptococcus faecalis*)
Nitrogen	2.1	4.6	10.1	5.6
Phosphorus	0.31	0.67	1.52	1.88
Lipid	8.5	9.2	22.6	2.3 *
Protein	13.0	27.0	60.0 †	
Glucan	28.8			
Mannan	31.0			
α-cellulose		15.4		
Hemicellulose		31.0		
Reducing value			16.0	61.0
Hexosamine	1–2	3.3	3.0	22.2

* Ether extractable material after HCl hydrolysis.
† An approximate figure.
References 12, 24, 29, 50.

stances encountered in walls from the main microbial groups are listed in Tables 5, 6, and 7.

Nature seems to have utilized the polysaccharides as the principle type of structural polymer. Some of these microbial wall polysaccharides have been identified as chitin [$\beta(1 \rightarrow 4)$ N-acetylglucosaminide] and cellulose. That both cellulose and chitin can occur together has been clearly established by Fuller and Barshad.[6] Both types of polysaccharides were found in the cell wall of the aquatic Phycomycete, *Rhizidiomyces* sp. It is now evident from studies of the chemistry of cell walls that although the walls of a

TABLE 5

Chemical Constituents of Microbial Cell Walls

Green Algae	
Chlorella pyrenoidosa	Polysaccharide, protein, and lipid
Platymonas subcordiformis	Polysaccharide * (galactose, uronic acid)
Gonyaulax polyedra	Polysaccharide (glucose)
Dunalliella	Lipo-protein (membrane ?)
Diatoms e.g., *Phaeodactylum*	Silica, polysaccharide
Red and Brown Algae	Polysaccharides (glucose, xylose, arabinose, uronic acids)

* Traces of amino acids.
References 29, 51–56.

TABLE 6

Chemical Constituents of Microbial Walls

Fungi	
Penicillium spp.	Polysaccharide (glucosamine,
Aspergilus spp.	Polysaccharide glucose, galactose, mannose) *
Rhizopus stolonifer	Polysaccharide (glucosamine)
Tricophyton mentagrophytes	Polysaccharide (glucosamine)
Neurospora crassa	Polysaccharide (glucose, glucosamine) *
Yeasts	
Saccharomyces cerevisiae	Polysaccharide, protein, lipid
Candida albicans	Polysaccharide, protein
Candida pulcherrima	Polysaccharide, protein

* Amino acids detectable.
References 5, 24–28, 57, 58.

TABLE 7

Chemical Constituents of Microbial Walls

Bacteria	
Eubacteria	
Gram-positive	Mucocomplex (mucopeptides, mucopolysaccharides) and teichoic acids
Gram-negative	Protein, polysaccharide, lipid, mucocomplex constituents
Myxobacteria	
Myxococcus xanthus	Protein, lipid, polysaccharides, mucopeptides, carotenoids
Blue-Green Algae	
Anacystis nidulans, *Microcoleus vaginatus*	Mucopeptide constituents, carotenoids
Nostoc sp.	Protein

References 45, 48, 50, 61–63

number of microorganisms are predominantly polysaccharide they contain in addition significant protein and lipid constituents. Furthermore, the investigations of Nickerson and his colleagues [59,60] have shown that in the yeast wall glucans and mannans occur as protein complexes and that they are not present as simple polysaccharides.

Comparative studies of cell-wall chemistry have also established the presence of a new type of structural heteropolymer, the mucocomplexes,[61] in walls of all bacteria so far examined, and in many of the Gram-positive bacteria they constitute the entire wall. The essential similarity of this class of cell-wall substance to other mucopolysaccharides was first pointed out as a result of the investigations of the wall of *Streptococcus faecalis* (Salton [12]), and their distinction from known mucoproteins was also emphasized. This

became even more evident when it was discovered that the wall of *Micrococcus lysodeikticus* was composed solely of hexosamine, glucose, and the four amino acids: alanine, glutamic acid, glycine, and lysine (Salton [50]). The mucocomplexes can be separated into further groups, depending on whether they are predominantly peptide, as in mucopeptides, or predominantly polysaccharide, as in mucopolysaccharides. (See Table 7.) In addition to the mucopeptides and mucopolysaccharides, Baddiley, Buchanan, and Carss [62] discovered that some bacterial cell walls also contain major components of ribitol- and glycerolphosphate polymers. These polymers have been called the "teichoic acids" (from Greek τεῖχος = wall) by Armstrong, Baddiley, Buchanan, Carss, and Greenberg.[63]

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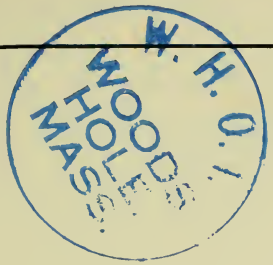
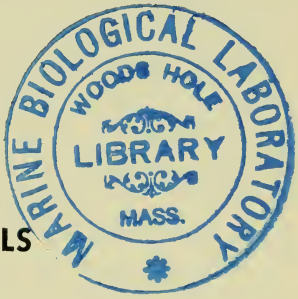
CHAPTER

2





CHEMISTRY OF CELL WALLS

Now we shall turn to the more detailed studies of the chemical constituents of microbial cell walls. For this discussion our selection of material is confined almost exclusively to yeast and bacterial cell walls. Some ten years ago very little was known about the chemistry of the walls of bacteria. This situation has been rapidly changed so that more is known about the chemical constitution of walls of bacteria than those of any other microorganism, and only a condensed account of the chemistry of bacterial walls can now be given in a single lecture.

Chemistry of Yeast Cell Walls

Long before the yeast wall had been isolated as a single morphological entity yeast polysaccharides had been purified and their structures investigated. Glucan from *Saccharomyces cerevisiae* and from *Candida albicans* both contain $\beta(1 \rightarrow 3)$ and $\beta(1 \rightarrow 6)$ glycosidic linkages, but the

polysaccharide from the latter appears to be more highly branched.[1,2,3,4] Some differences in the linkages and degree of branching have been suggested for mannans derived from various yeasts.[4,5,6]

Isolation of walls by mechanical disintegration led to the discovery of protein and lipid components in addition to the polysaccharides.[7,8,9,10] Not all yeast species contain appreciable quantities of lipid in the wall, for Kessler and Nickerson [9] found as little as 1% total lipid in the walls of strains of *Candida albicans* and as much as 10% in the wall of *Saccharomyces cerevisiae*. A clearer understanding of the molecular architecture of the yeast cell wall has begun to emerge from the important discovery by Falcone and Nickerson [8] that the wall polysaccharides occur as protein complexes. Further investigations by Kessler and Nickerson [9] have established the presence of a glucan-protein complex and two types of glucomannan-protein complexes in a variety of yeast walls. The percentage of the wall accounted for by the various polysaccharide-protein complexes for several yeasts is illustrated in Table 8. The presence of a mannan-protein complex in baker's yeast wall has been confirmed by Korn and Northcote,[11] and, from alterations in the surface charge of yeast walls degraded with various enzymes, Eddy [12] has suggested that the mannan-protein complex forms part of the outside layer of the wall. However, this suggestion, based on microelectrophoresis data, must await more definitive biochemical and chemical investigations. The nature of the bonding between the polysaccaride-protein complexes is not known, but Kessler and Nickerson [9] suggest the possibility of esterification of carboxyl groups of the protein with hydroxyl groups of the polysaccharides.

TABLE 8

The Percentages of Various Polysaccharide-Protein Complexes in the Walls of Several Yeasts *

Organism	Recoveries of Cell Wall Complexes		
	Glucan Protein %	Glucomannan-Protein I %	Glucomannan-Protein II %
Baker's yeast	41.6	13.6	34.7
Saccharomyces cerevisiae 18.29	28.3	55.8	11.9
Candida albicans RM806	46.7	7.5	41.5
Candida albicans 582	47.4	3.0	27.2

* From the data of Kessler and Nickerson.[8]

Chemistry of Bacterial Cell Walls

In discussing the chemistry of bacterial cell walls, it becomes necessary to distinguish between the two major groups of organisms differentiated by the Gram stain reaction. Early comparative studies of wall composition indicated the greater complexity of the walls isolated from Gram-negative bacteria.[13,14] This chemical heterogeneity was later found to be paralleled by a more complicated physical structure of multilayered walls with macromolecular subunits, as mentioned in Chapter 1. Whether the greater chemical complexity of the "wall" of Gram-negative bacteria is due to the presence of a single structure possessing the functional units of a true wall and a membrane has not been satisfactorily resolved. However, the thin sections

of Gram-negative bacteria give strong support to the belief that there are indeed two separate structures, a complex wall and a membrane.[15,16,17] (See Fig. 7.) It appears likely, then, that the differences in wall composition between the Gram-positive and Gram-negative bacteria amply demonstrated in many investigations [14,18,19] are real and are not an artifact of a major structural difference between the two groups of organisms. What is worth emphasizing here is that both groups of organisms possess mucopeptide constituents in common, a finding that has led to the idea of a "basal" structure being present in all bacterial cell walls (Work [20]). The nature of the basal structure has become more apparent, and it is likely that one of a variety of mucopeptides can perform this function.[21,22] What is uncertain at the moment is the variety of monomeric constituents in the mucopeptides from both Gram-positive and Gram-negative bacterial walls. At the present time there is insufficient evidence to suggest that the term "basal structure" means any more than a class of mucopeptides containing some common building units of amino sugars and amino acids.

Constituents of Walls of Gram-Positive Bacteria

Analysis of the walls of Gram-positive bacteria revealed the presence of both nitrogen and phosphorus, and in *Bacillus subtilis* walls the content of P was very high.[13] On hydrolysis the walls contained reducing substances and amino sugars, and some of the typical results are shown, together with N and P determinations, in Table 9.

The first fascinating detail to emerge from the early studies of cell-wall chemistry was the small variety of amino acids in the walls of some bacteria.[13] Thus the wall of *Micrococcus lysodeikticus* isolated by mechanical disintegration and receiving no treatments other than washing with

TABLE 9

Composition of the Walls of Several Gram-Positive Bacteria

	% N	% P	% Reducing substances	% Amino sugar
Bacillus megaterium	5.3	0.42	48	18
Bacillus subtilis	5.1	5.35	34	8.5
Micrococcus lysodeikticus	8.7	0.09	45	16
Sarcina lutea	7.6	0.22	46	16
Streptococcus faecalis	5.6	1.88	61	22

Reference 13.

M NaCl and water contained the four amino acids (alanine, glutamic acid, glycine, and lysine) together with hexosamine and glucose.[13] Since then a great deal of qualitative and quantitative work on walls has been performed by Cummins and Harris,[23,24] Work,[20] Snell, Radin, and Ikawa,[25] Ikawa and Snell,[26] Strange,[27] Baddiley, Buchanan and Carss,[28] Armstrong, Baddiley, Buchanan, Carss, and Greenberg,[29] and Abrams,[30] and the following oustanding features have firmly established some of the characteristic chemical properties of the walls:

(*a*) Variety of principal amino acids limited to 3, 4, or 5.

(*b*) Discovery of diaminopimelic acid in certain microorganisms and its localization in the wall.

(*c*) The detection of D-isomers of amino acids.

(*d*) The isolation and characterization of the amino sugar, muramic acid, from spore peptides and walls.

(*e*) The detection of ribitolphosphate polymers in walls and the discovery of the teichoic acids.

(*f*) The detection of O-acetyl groups.

(*g*) The presence of ester-linked alanine.

The identification of the principal constituents of the walls of Gram-positive bacteria has led to the conclusion that the walls belong to the general class of chemical compounds known as mucocomplex substances.[13,14,19,20,31,32] These mucocomplex polymers can be further subdivided, depending on whether peptide components predominate or whether they are predominantly polysaccharide in nature as below:

mucopeptides—composed of amino acids and amino sugars
mucopolysaccharides—sugars and amino sugars

It is probable that in some cell walls both are covalently joined so that soluble wall compounds derived either chemically or enzymically may be essentially either mucopeptide or mucopolysaccharide but containing minor residues of one or the other. In addition to these two classes of substances, we must now add the teichoic acids [28,29] as major wall compounds. The walls of Gram-positive bacteria may therefore be wholly mucopeptide [33,34] or predominantly mucopolysaccharide, with smaller amounts of mucopeptide as in some streptococcal walls,[14] or they may contain mucopeptides, mucopolysaccharides, and teichoic acids.

Amino-Acid Composition. The distribution of major amino acids has been studied in some detail by Cummins and Harris.[23,24,31] Amino acid constituents, and in some cases the monosaccharide components of walls, have been of great taxonomic value.[31,32] The principal combinations of the major amino acids found in walls are presented in Table 10. It will be seen that in none of the walls so far

TABLE 10

Principal Combinations of Major Amino Acid Constituents Found in Walls of Gram-Positive Bacteria

Groups	Amino Acids
Staphylococci Micrococci	Alanine, glutamic acid, lysine, glycine, and serine in some
Streptococci Lactobacilli Aerococci	Alanine, glutamic acid, lysine, and aspartic acid in some
Bacilli Coynebacteria Mycobacteria Nocardia	Alanine, glutamic acid, DAP
Micrococci Clostridia Proprionibacteria Streptomyces Micromonospora	Alanine, glutamic acid, DAP, glycine

References 23, 24, 31, 37, 41, 51

studied do diaminopimelic acid (DAP) and lysine occur together as major amino acid constituents.

More and more information on the quantitative amino acid composition of bacterial walls has become available (Perkins and Rogers,[33] Rogers and Perkins,[34] Strominger, Park, and Thompson,[35] Hancock [36]), and on the whole there is good agreement for various organisms, although it is now apparent that there will be significant differences between various strains.[34] The molar ratios of the principal amino acids of walls from various species investigated by Salton

and Pavlik [37] are presented in Tables 11 and 12. It is evident from these results that the peptide composition may vary widely from one group to another, although in some

TABLE 11

Relative Molecular Proportions of the Principal Amino Acids in Cell Walls

Walls from	Lysine	Glutamic Acid	Glycine	Serine	Alanine
Bacillus sp.*	1	1.7	0.5	0.3	2.3
Corynebacterium sp.	1	1.0	0 †	0.7	3.9
Micrococcus citreus	1	3.0	0.8	0	2.1
Micrococcus lysodeikticus	1	1.0	1.0	0	2.6
Micrococcus roseus	1	1.1	0	0	5.1
Micrococcus tetragenus	1	1.2	1.2	0	2.3
Micrococcus urea	1	1.3	1.0	0	2.3
Sarcina flava	1	1.4	1.0	0	2.2
Sarcina lutea	1	1.6	1.0	0	2.0
Sporosarcina ureae	1	1.7	1.0	0	2.0
Staphylococcus albus	1	1.1	4.8	0.4	2.9
Staphylococcus aureus	1	1.1	4.8	0.45	3.0
Staphylococcus citreus	1	1.0	4.0	0.5	3.1
Staphylococcus saprophyticus	1	1.0	4.6	0.6	3.3
Streptococcus faecalis	1	0.9	0	0	4.0

* A high proportion of threonine (0.7) was also present.

† "0" used to designate absence or only faint traces of amino acids.

Reference 37, N. B. Serine values for the four staphylococci incorrectly shown in Salton and Pavlik [37] have been corrected above.

TABLE 12

Relative Molecular Proportions of the Principal Amino Acids in Cell Walls

Walls from	DAP *	Glutamic Acid	Glycine	Alanine
Bacillus cereus	1	1.3	0	2.6
Bacillus megaterium	1	1.8	0	2.8
Bacillus pumilis	1	1.6	0	4.6
Bacillus stearothermophilus	1	2.0	0	3.8
Bacillus subtilis	1	2.4	0	4.3
Bacillus thuringiensis	1	1.4	0	2.8
Micrococcus varians	1	4.3	1.8	2.6
Lactobacillus arabinosus	1	1.1	0	2.9

* α,ϵ-diaminopimelic acid.
Reference 37.

instances very similar ratios of amino acids were observed. No one would claim that these results represent anything more than the gross amino acid composition of the walls, as they give no indication of the distribution in the various wall components such as the mucopeptides and teichoic acids or other special structures that may yet remain to be discovered.

Apart from the peptide residue of the nucleotide isolated from penicillin-inhibited *Staphylococcus aureus* (Park,[38] Park and Strominger [39]), there are no published accounts of the sequence of amino acids in peptides derived directly from cell walls.

In addition to the known amino acids, bacterial walls have yielded upon hydrolysis several unknown ninhydrin-reacting constituents.[13,23,24,37] Cummins and Harris [23]

found an unknown compound in the walls of lactobacilli. This substance was found to be a peptide of lysine and aspartic acid (α-aminosuccinoyllysine), which was more resistant to acid hydrolysis. The compound in which the aspartic acid is joined to the ϵNH_2 group of lysine was also encountered in hydrolysates of the antibiotic bacitracin.[40]

Appreciable amounts of ammonia have been found on hydrolysis of cell walls by Ikawa and Snell,[41] and if this is not due simply to destruction of wall compounds such as the amino sugars it indicates the possibility that some amino acids may be present as amides. Typical results for the amino acid composition of several lactic acid bacteria from the studies of Ikawa and Snell [41] are presented in Table 13.

TABLE 13

Amino Acid Composition of Walls from Lactic Acid Bacteria *

	(mg per 100 mg cell wall)		
	Streptococcus faecalis	*Lactobacillus plantarum*	*Lactobacillus citrovorum*
Glutamic acid L	0.6	0	0.9
D	4.6	7.6	10.4
Alanine (total)	4.4	11.6	9.8
D	1.7	3.7	4.6
Aspartic acid (total)	2.4	0.6	8.1
L	0.7		1.8
Lysine (total)	2.5	0.4	5.6
L	2.4	0.5	6.2
DAP	0	5.2	0
α-aminosuccinoyllysine	0	0	4.4
Ammonia	1.1	2.4	3.3

* Data from Ikawa and Snell.[41]

Occurrence of D-isomers of Amino Acids. Snell and his colleagues [25,26] were the first to discover that the D-alanine found in bacterial cells was localized in the wall. A high proportion of the cell-wall alanine was present as the D-isomer. Glutamic acid was subsequently found in the wall as the D-isomer.[26] Salton [42] also showed that D-alanine occurred quite widely in the walls of various bacterial species. The list of D-amino acids in bacterial walls was extended to aspartic acid when Toennies, Bakay, and Shockman [43] found that this amino acid occurred partly as the D-isomer in the wall of *Streptococcus faecalis.* Ikawa and Snell [41] have made an extensive investigation of the proportions of D- and L-isomers of alanine, glutamic, and aspartic acids in the walls of many lactic acid bacteria, and some of the results are summarized in Table 14. Thus about half of the cell-wall alanine occurs as the D-isomer and virtually all of the glutamic acid is in the D-form, whereas D-aspartic acid residues constitute roughly three quarters of the total aspartic acid contents. Park [44] has observed that many walls have 1:1 ratios of D-glutamic acid to muramic acid.

Evidence so far available suggests that only L-lysine is present in walls.[41] However, DAP can occur in bacteria as the LL-, meso(DL)-, or DD-isomers, and occasionally the LL- and meso-isomers together (Hoare and Work [45]). The meso-isomer is most widely distributed in bacteria and the isolated walls, being found in members of the Bacillus, Corynebacterium, Mycobacterium, Nocardia, and, less frequently, in certain species of Lactobacillus and Micrococcus groups.[19,20,24,37,45] LL-DAP has been detected in members of the Propionibacterium, Streptomyces, and some Clostridium species.[24,45] Hoare and Work [45] found some DD-isomer of DAP in hydrolysates of Micromonospora, the presence of this isomer in isolated walls being confirmed later.[24]

TABLE 14

Percentage of Glutamic Acid, Aspartic Acid, and Alanine in the D-configuration in Cell Walls

	(% of total in D-form)		
	Glutamic Acid	Aspartic Acid	Alanine
Streptococcus faecalis	85	71	39
Lactobacillus casei	100	50	61
Lactobacillus plantarum	100		32
Lactobacillus mesenteroides	73	67	54
Lactobacillus pentosus	94		66
Lactobacillus citrovorum	89	78	47
Lactobacillus bulgaricus	87	72	40
Lactobacillus lactis	94	78	61
Lactobacillus acidophilus	91	67	48

* Data from Ikawa and Snell.[41]

Identification of N-Terminal and C-Terminal Amino Acids. Attempts to apply some of the classical techniques for determining the chemical structure of proteins to bacterial cell walls have been complicated by some of the unusual features of cell-wall composition [see foregoing (*a*)–(*g*)]. The cell-wall constituents possessing free amino groups can be readily identified by reacting the walls with 1-fluoro-2,4-dinitrobenzene (FDNB),[46] the method introduced by Sanger [47] for the determination of the N-terminal residues in proteins. However, the interpretation of results with bacterial walls is complicated by the presence of ester-linked alanine in the teichoic acids.[29] O-alanyl residues would thus behave as N-terminal amino acids. With walls from *Micrococcus lysodeikticus* and *Sarcina lutea* that are

devoid of the teichoic acids, the DNP-alanine detectable on reaction with FDNB probably represents the N-terminal residue of the wall peptides, and from this data a subunit size can be tentatively suggested.[46] The contribution of the teichoic acids to the N-terminal alanine residues can be surmised from a comparison of the amounts of DNP-alanine obtained from the walls of *Micrococcus lysodeikticus* (23 μM/g) with those of *Staphylococcus aureus* (170 μM/g) and *Lactobacillus arabinosus* (120 μM/g), both rich in teichoic acids.[48] The relatively small number of N-terminal groups in walls other than those containing large amounts of teichoic acids is perhaps not surprising, as the "free" amino groups of peptides would be required for amide bonding to muramic acid. The comparatively low yields of N-terminal residues could, of course, be equally well explained by cyclic peptide structures or N-acetylation of amino acid residues.

The application of carboxypeptidase for the identification of C-terminal residues of walls of Gram-positive bacteria has not been successful (Perkins and Rogers,[33] Salton [49]). This is not at all surprising, since the cell-wall peptides contain D-isomers of several amino acids. Hydrazinolysis,[50] on the other hand, has been much more successful, and with some walls this method has given very clean results, although their interpretation poses several interesting problems of the molecular structure of walls. The yields of C-terminal amino acids (uncorrected for any losses during hydrazinolysis) from several cell walls and lysozyme-digest products are given in Table 15.

Whether we are really dealing with C-terminal residues in the protein sense (i.e., at the end of a peptide chain) is not known. It is conceivable that special types of linkages of amino acids in the wall peptides could give false "C-terminal" values in just the same way as O-alanyl groups behave

TABLE 15

C-terminal Amino Acids of Bacterial Cell Walls Determined by Hydrazinoylsis

	(μM/10 mg cell wall *)			
	Glycine	Glutamic Acid	Alanine	DAP
Sarcina lutea	1.84	0.47	<0.1	—†
Micrococcus lysodeikticus				
Prep. 1	2.47	0	0.21	—
Prep. 2	2.52	0	0.37	—
Lysozyme-NDF ‡	4.1	0	0.21	—
Bacillus megaterium	—	<0.1	0.35	1.29
Micrococcus varians	<0.1	<0.1	<0.1	0.14

* Values uncorrected for possible losses during reaction.
† Amino acid not present in these walls.
‡ Nondialyzable fraction from lysozyme-digested walls.
Data from Salton.[48]

as false "N-terminal" residues. If amino acids occurred as single substituents on muramic acid, or in the form of a side chain linked to the γ-carboxyl group of glutamic acid, they would also behave as C-terminal substances. The only evidence so far available supporting this suggestion is the report by Perkins and Rogers [33] that a diffusible compound in partial acid hydrolysates of *Micrococcus lysodeikticus* walls possessed muramic acid, glucosamine, and glycine in equimolar proportions. The occurrence of substituent groups of glycine on some muramic acid residues would be compatible with the large number of C-terminal groups found in the wall of this organism (see Table 15) and could also explain the origin of free glycine in walls digested with the Streptomyces enzyme complex (Salton and Ghuysen [51])

now known to contain an amidase capable of acting on small molecular weight mucopeptides (Ghuysen [52]). Thus in the walls of *Micrococcus lysodeikticus* the number of C-terminal glycine groups could be due to special groupings on the wall mucopeptide or could represent the true ends of the peptide chains. If the latter, a subunit size of approximately 4000 molecular weight suggests that this wall possesses relatively short peptide chains on the amino sugar backbone.[48]

Amino Sugar Constituents. The key to understanding the structure of the bacterial cell-wall mucopeptides and mucopolysaccharides was provided by the detection and isolation of a new acidic amino sugar by Strange [27] and his colleagues. This amino sugar, now known as muramic acid, was first found in the spore peptides isolated by Strange

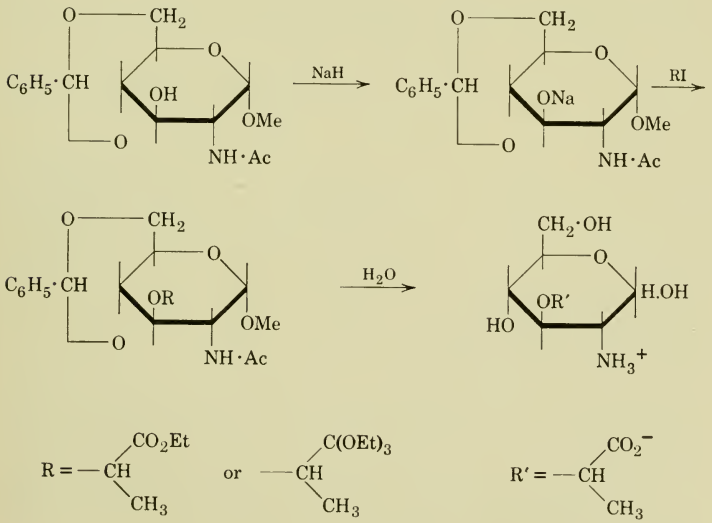


Fig. 8. Synthesis of muramic acid.

and Powell [53] and was subsequently found in bacterial cell walls.[23, 54, 55, 56] The unknown amino sugar in the nucleotides accumulating in penicillin-treated *Staphylococcus aureus* discovered by Park and Johnson [57] in 1949 was later found to be identical to the cell-wall amino sugar.[39]

Muramic acid (3-O-carboxyethyl-D-glucosamine) was isolated as a crystalline substance by Strange and Dark,[55] and the structure was established by the synthetic route worked out by Strange and Kent,[58] starting with the N-acetyl-4:6-O-benzylidene-α-methyl-D-glucosaminide, as shown in Fig. 8.

Some of the properties of natural and the synthetic stereoisomers of muramic acid are summarized in Table 16. The

TABLE 16

Optical Rotation and Chromatographic Behavior of Natural and Synthetic Muramic Acid and the Synthetic Isomer

	Average Values Derived from Several Experiments		
	Optical Rotation $[\alpha]_D^2$	R_F *	$R_{\text{glucosamine value}}$ † on Zeo-Karb 225 Column Eluted with 0.33 N-HCl
Natural muramic acid	+109	0.53	1.10
Synthetic muramic acid	+109	0.53	1.10
Stereoisomer of muramic acid	+ 52	0.44	0.87

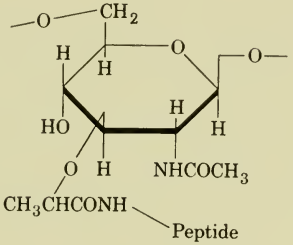
* Values obtained with Whatman No. 1 paper and phenol-water as solvent.

† Values in this column have been reported by Crumpton (1958). The $R_{\text{glucosamine value}}$ relates the elution characteristics of the substance to those of glucosamine run at the same time.

Reference, Strange, and Kent.[58]

comparison of the optical rotations of the synthetic and naturally occurring compound suggests that the lactic acid residue of the spore-peptide muramic acid possessed the D-configuration. Zilliken [59] has confirmed the synthesis of muramic acid from D-glucosamine, using several modifications to the procedure developed by Strange and Kent.[58]

The structures proposed for muramic acid and that of the muramic acid-nucleotide from *Staphylococcus aureus* [39,58] suggest that the general function of muramic acid in the cell wall is to link peptides (through an amide bond at the carboxyl group of muramic acid) to other amino sugar or sugar residues as shown below:



Although Park [44] has shown that there is a 1:1 ratio of muramic acid to D-glutamic acid in the walls of a number of bacteria, it should not be assumed from the general type of structure previously suggested that all or nearly all of the muramic acid residues have peptide substituents. As is shown in Chapter 3, in *Micrococcus lysodeikticus* walls much of the muramic acid is unsubstituted. However, it is not difficult to visualize that in some bacterial walls (possibly those resistant to lysozyme) peptides may form a cross link between parallel chains of amino sugar oligosaccharides, being linked through muramic acid at each end of the peptide. This could be especially the case with those walls containing DAP (or lysine), as there is evidence that both

amino groups of DAP may be unavailable for reaction with FDNB in a high proportion of the residues in some cell walls.[46]

Muramic acid has been detected (usually by paper chromatography) in all of the bacterial cell walls so far examined.[19,21,22,24,42] Whether the structures are identical in all cases and whether all muramic acids are the 3-O-D-lactyl ethers of glucosamine remains to be established. It is of interest to note that Ågren and Verdier[60] have isolated 6-phosphoryl muramic acid from a protein-bound compound in *Lactobacillus casei*. It will be of great interest to learn whether this compound occurs in the wall as the phosphoryl derivative.

In addition to muramic acid, glucosamine is also universally present in bacterial cell walls.[13,14,19] Galactosamine has been found, together with muramic acid and glucosamine, in some bacterial walls, but it seems to be much less widely distributed.[19,23,24,31] It is probable that all three amino sugars occur in the walls as N-acetyl or as N-acyl compounds. The reaction of walls with FDNB has so far shown that none of the amino groups of the amino sugars is free.[46]

Monosaccharides. Some bacterial walls are composed entirely of amino acids and amino sugars being devoid of other sugar components.[31,37] However, many bacterial walls yield monosaccharides on hydrolysis, and the investigations of Cummins and Harris[23,24,31] have shown that the sugar components are characteristic of certain taxonomic groups. Glucose occurs commonly in many bacterial walls and, as will be seen later, it may also be a constituent of the teichoic acid moiety of the wall. Rhamnose, first found as a wall monosaccharide in *Streptococcus faecalis*,[61] is the typical sugar of the streptococcal group. Arabinose, detected in the wall and isolated cell-wall oligosaccharide of

Corynebacterium diphtheriae by Holdsworth,[62] was subsequently found to be confined to a number of related groups. Some of the monosaccharides characteristic of various bacterial groups are presented in Table 17.

The occurrence of mucopolysaccharides in the walls of some bacteria is supported by the isolation of the oligosaccharide from *Corynebacterium diphtheriae* by Holdsworth.[62] Fairly drastic conditions were required for the liberation of the oligosaccharide from the cell-wall muco-

TABLE 17

Principal Combinations of Monosaccharide Constituents Found in Walls of Gram-Positive Bacteria

Groups	Sugars
Staphylococci Sporosarcina Streptomyces	None
Staphylococci Micrococci Aerococci Bacilli Streptomyces	Glucose, galactose, mannose (singly or in combination)
Streptococci Lactobacilli Propionibacteria Clostridia	Rhamnose, glucose, galactose, mannose (Rhamnose alone or in combination with Rhamnose)
Corynebacteria Mycobacteria Nocardia	Arabinose, glucose, galactose, mannose (in combination with arabinose)

References 22, 23, 24, 31, 37, 62, 63, 64.

complex,[62] suggesting a firm chemical combination between the polysaccharide and the rest of the wall (as for *Streptococcus faecalis* [61]). Further evidence establishing the presence of mucopolysaccharides in the walls has come from the investigation by McCarty [65] of the products of enzymic digestion of Group A streptococcal walls. The "C" carbohydrate fractions from the streptococcal wall still contained small residues of peptide but were composed predominantly of amino sugar and rhamnose.[65] Further fractionation failed to remove the peptide constituents, and there seems little doubt that these mucopolysaccharides were joined to the mucopeptides in the original wall.

Teichoic Acids. Mitchell and Moyle [66] reported the presence of a polyglycerophosphate compound in the envelope of *Staphylococcus aureus,* and the status of this material as a wall component remained uncertain until the problem was taken up again following the discovery by Baddiley and his colleagues [67] of the two nucleotides, cytidine diphosphoglycerol and cytidine diphosphoribitol. It will be recalled that the wall of *Bacillus subtilis* had a very high phosphorus content (see Table 9), and it was not surprising that an examination of the wall of this organism and that of *Lactobacillus arabinosus* [syn. *Lactobacillus plantarum*] (the organism from which the two nucleotides were isolated) revealed the presence of ribitolphosphate polymers.[28] No glycerophosphate polymer was detectable in the walls of either of these organisms. The name teichoic acids was given originally only to the ribitolphosphate polymer,[29] but since the confirmation of the presence of a glycerophosphate polymer in walls of other bacteria, and the detection of both types in yet others, the term teichoic acids has been extended to include both types of polyols.[68] The distribution of the two types of teichoic acids in various cell walls

has been studied by Armstrong et al.[68] and is presented in Table 18.

Glycerophosphate polymers have been detected in a number of Gram-positive bacteria by McCarty,[69] but he was unable to find these localized in the walls. These polymers thus probably differ from the glycerol type of teichoic acid, which in common with the ribitol teichoic acids contain O-alanyl groups.[68]

The teichoic acids can be extracted from the isolated walls with trichloroacetic acid (TCA), and Armstrong et al.[29] suggest that they may be bound to the other wall constituents by salt linkages. However, conditions for extraction with

TABLE 18

Distribution of Teichoic Acids in Bacterial Cell Walls

	Type of Polymer	
	Glycerol	Ribitol
Lactobacillus arabinosus 17-5	−	+
Lactobacillus casei (A.T.C. 7469)	+	−
Lactobacillus delbrückii (N.C.I.B. 8608)	+	−
Lactobacillus bulgaricus (N.C.I.B. 76)	+	−
Staphylococcus aureus H	trace	+
Staphylococcus aureus (Duncan)	trace	+
Staphylococcus aureus (Oxford)	+	+
Staphylococcus citreus	+	−
Staphylococcus albus (N.C.T.C. 7944)	+	−
Bacillus subtilis (vegetative form)	−	+
Escherichia coli Type B	trace	−
Corynebacterium xerosis	+	−
Streptococcus faecalis (A.T.C. 9790)	+	+

Reference 68.

TCA are hydrolytic,[37,48] and the mode of attachment of the teichoic acids remains uncertain at present. The products of acid hydrolysis of the teichoic acids removed from walls by extraction with TCA have been examined by Armstrong et al.,[29] and Table 19 illustrates the variety of compounds detectable in the ribitol type; 1:4 anhydroribitol is one of the main products detectable, but, as pointed out by Salton and Pavlik,[37] in 6*N* hydrochloric acid hydrolysates of walls a faster-moving component (possibly dianhydroribitol), not previously reported, is detectable on paper chromatograms.

One of the interesting features of the structure of the teichoic acids was the discovery of ester-linked alanine, the first reported occurrence of this type of linkage of an amino acid in a natural product. The detailed structure of the ribitol teichoic acid from *Bacillus subtilis* has been proposed

TABLE 19

Products of Acid Hydrolysis of Teichoic Acid from Different Bacteria

	Lactobacillus arabinosus	*Bacillus subtilis*	*Staphylococcus aureus*
Alanine	+	+	+
Glucose	+	+	−
Glucosamine	−	−	+
Inorganic phosphate	+	+	+
Anhydroribitol	+	+	+
Anhydroribitol phosphate	+	+	+
Ribitol	+	+	+
Ribitol glucosaminide	−	−	+

Reference 29.

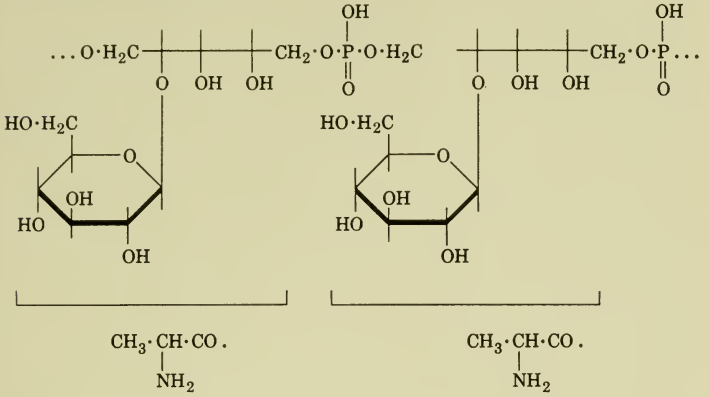
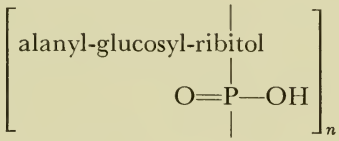
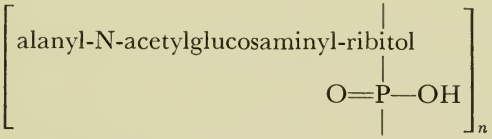
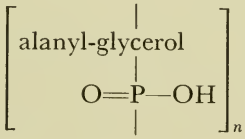


Fig. 9. The structure of teichoic acid from the cell walls of *Bacillus subtilis.*

by Armstrong, Baddiley, and Buchanan,[70] as shown in Fig. 9, and the three general types of teichoic acid are represented in formulas 1, 2, and 3.[68]

(1)


(2)


(3)


O-ester Groups. The presence of O-substituents in bacterial walls was first reported by Abrams,[30] who discovered O-acetyl groups in the walls of *Streptococcus faecalis* and those of several other bacteria. Brumfitt, Wardlaw, and Park [71] subsequently found that a lysozyme-resistant mutant of *Micrococcus lysodeikticus* contained a much greater amount of O-acetyl in the walls than the parent strain. Removal of the O-acetyl groups restored the sensitivity to lysozyme. The O-alanyl groups of the teichoic acids are the only other O-ester groups so far reported in bacterial walls.

Whether the teichoic acids in *Lactobacillus arabinosus* are ester linked to other wall components is not known, but it is of interest to note that the lysozyme sensitivity of the isolated walls of this organism is greatly increased after extraction with reagents removing O-esters and/or teichoic acid.[37] Armstrong et al.[29] reported that alanine was the only O-ester in the wall of this organism, so it appears that the change in lysozyme sensitivity does not involve removal of O-acetyl groups as in *Micrococcus lysodeikticus* walls.[71]

Composition of Walls of Gram-Negative Bacteria

The status of our knowledge of the chemistry of the walls of Gram-negative bacteria is less satisfactory, although a clearer picture is beginning to emerge from the detailed studies of *Escherichia coli* walls by Weidel and his colleagues. The greater complexity of the walls of Gram-negative bacteria has already been emphasized.[13,14] In addition to a complete range of amino acids, they also contain substantial amounts of lipid and frequently a variety of monosaccharide constituents. The amino sugar contents are generally lower than those found for the majority of walls from Gram-positive bacteria. Some typical analyses for amino sugar contents and amounts of lipid in the walls of

a number of Gram-negative bacteria are given in Table 20.

One of the most important recent developments in the study of the chemistry of the walls of Gram-negative bacteria has been the recognition of mucopeptide constituents of a similar nature to those forming the whole cell-wall structure of Gram-positive bacteria. This discovery has followed from a number of investigations on the occurrence of DAP [20] and the detection of both DAP and muramic acid in the walls of *Escherichia coli* [21,72] and those of a variety of Gram-negative bacteria.[13,42] Furthermore, Park [44] reported the presence of D-glutamic acid in *Escherichia coli* walls, and a small amount of D-alanine was detected in the wall of *Rhodospirillum rubrum*.[42] Additional evidence for the existence of the mucopeptide in *Escherichia coli* wall came from the work of Weidel and Primosigh [21,72] when they discovered that the phenol-insoluble fraction of the wall con-

TABLE 20

Lipid and Amino Sugar Contents of the Walls of Gram-Negative Bacteria

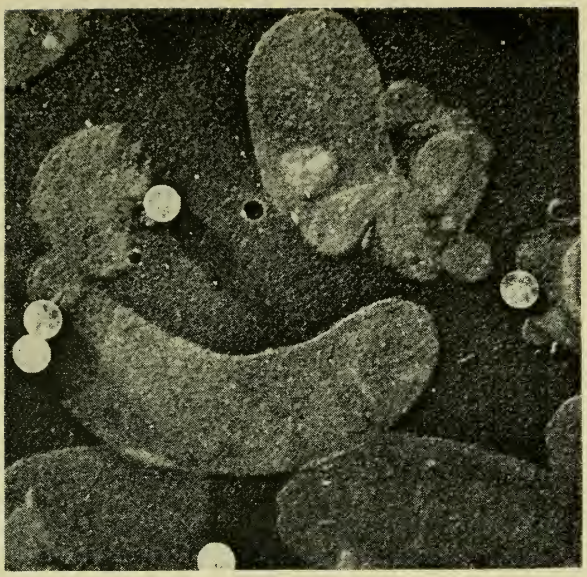
	% Dry Weight Cell Wall	
	Total Lipid	Amino Sugars
Escherichia coli	22	3.0
Salmonella pullorum	19	4.8
Salmonella gallinarum	22	3.9
Vibrio metchnikovi	11	1.9
Pseudomonas aeruginosa	11	2.1–2.7
Rhodospirillum rubrum	22	2.0
Chlorobium thiosulphatophilum	20	4.2
Organism LC1	—	13.0

References 13, 14, 22.

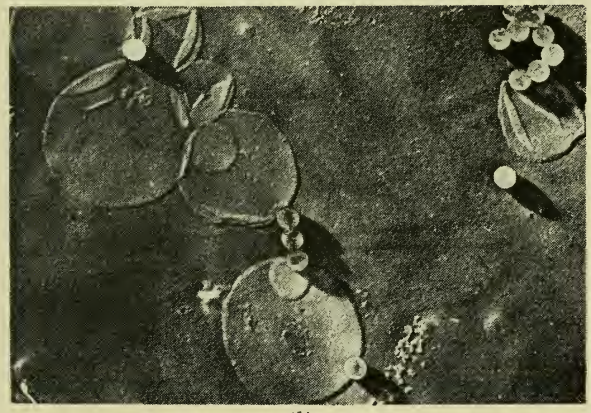
tained alanine, glutamic acid, DAP, glucosamine, and muramic acid as principal constituents. Material of this general composition was released from the wall on treatment with T_2 bacteriophage enzyme. Salton [22] showed that all but traces of the cell-wall DAP and muramic acid were released into the soluble fraction when lysozyme acted on the isolated walls of several Gram-negative bacteria, including *Escherichia coli*. The composition of the soluble nondialyzable constituents released by lysozyme from the walls of the Gram-negative bacteria showed that again alanine, glutamic acid, DAP, and glucosamine were predominant constituents with smaller amounts of muramic acid.[22]

There seems little doubt now that the mucopeptide is the component that is responsible for the structural rigidity of the walls of Gram-negative bacteria, although it may account for as little as 10 to 20% of the weight of the wall.[14, 21, 22] That the loss of the mucopeptide brings about a collapse of the rigid cell-wall structure has been directly demonstrated with isolated walls of *Rhodospirillum rubrum* by the author. Figure 10*a* shows the appearance of *R. rubrum* walls before treatment with lysozyme, and Fig. 10*b* shows how the structures become spherical on incubation with 100 μg lysozyme per milliliter under conditions giving a release of mucopeptide constituents.[22] The actual amounts of mucopeptide in the walls of Gram-negative bacteria probably vary from one species to another, and the data on amino sugar contents (Table 20) suggest that a whole spectrum of mucopeptide contents exists.[14]

The bulk of the wall of at least a number of Gram-negative bacteria is made up of protein, lipid, and polysaccharide complexes, undoubtedly forming the surface antigenic components. The cell walls isolated from Gram-negative bacteria contain the monosaccharide constituents that are characteristic of the purified lipo-polysaccharide



(*a*)



(*b*)

Fig. 10. (*a*) Isolated walls of *Rhodospirillum rubrum* (×20,500). (*b*) Walls of *R. rubrum* treated with lysozyme, showing conversion from the normal spiral fragments as in (*a*) to spherical structures (×11,500). M. R. J. Salton, unpublished.

antigens.[14,73] Thus, some of the dideoxyhexoses characterized by Westphal and his collaborators [74,75] and heptoses [76,77] are present in the bacterial walls. The characteristic spectra of the products of the Dische [78] reaction of heptoses have been used to show that these monosaccharides are located in the lysozyme-insoluble fraction of the wall, clearly indicating that they are not part of the mucopeptide structure.[73] A typical result for the walls of *Spirillum serpens* is shown in Fig. 11.

Much remains to be done in the investigation of the walls of Gram-negative bacteria, and at the moment we have no precise information about the number of different molecular or macromolecular subunits in the walls of this group. At least we are now certain that mucopeptides are common

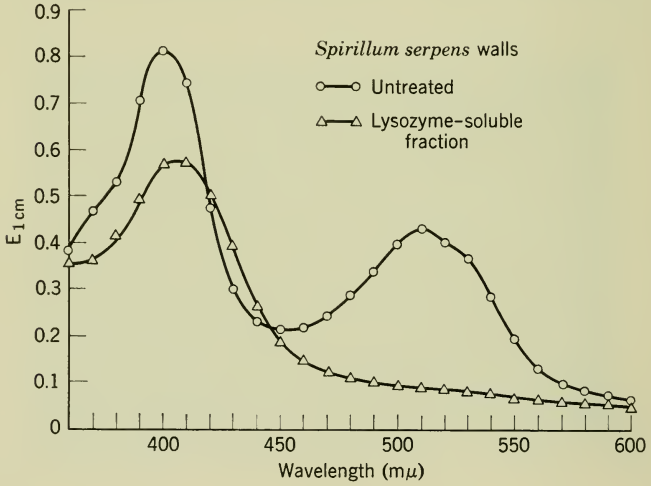


Fig. 11. Spectra of Dische reaction products of walls of *Spirillum serpens.*

to walls from both Gram-positive and Gram-negative groups of bacteria, and these can be identified by characteristic components such as DAP, muramic acid, and D-isomers of alanine and glutamic acid. However, the arrangement of the mucopeptide constituents in the walls of Gram-negative bacteria may differ in that they form a reinforcing network rather than a continuous sheet of the polymer. The fact that the isolated walls of Gram-negative bacteria can be completely disaggregated by sodium dodecyl sulfate strongly suggests this idea.[79]

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CHAPTER

3

ENZYMIC DEGRADATION AND BIOSYNTHESIS OF MICROBIAL WALLS

Enzymic Degradation

From some of the unusual features of the chemical properties of microbial cell walls outlined in Chapter 2 it is now easy to understand why they resist many of the proteolytic enzymes so active in degrading intracellular proteins and the various enzymes capable of breaking down lipids, polysaccharides, and other cellular constituents. The resistance of bacterial cell walls to proteolytic enzymes is especially conspicuous, and even if it was purely fortuitous that the D-isomers of amino acids were formed into wall peptides it seems eminently sensible that they should be there. Although many microbial walls are unattacked by enzymes degrading the intracellular structures and constituents, they can, as already pointed out, be attacked by enzymes produced by the cells themselves and by a variety of enzymes from other microorganisms and from other cells and tissues.

Algae, Fungi, Yeasts. The gut of the snail provides a collection of enzymes that have been used in degrading the

wall structures of the alga *Chlorella pyrenoidosa*,[1] *Neurospora crassa*,[2] and yeast.[3] Cellulases and chitinases in the snail-gut enzymes are generally believed to be active in cell-wall digestion, but most investigators have used unfractionated preparations undoubtedly rich in a variety of enzymes. Indeed, Myers and Northcote [4] reported that the snail enzyme preparations contained active lipases and carbohydrases, including cellulase, xylanase, and mannanase. Only weak proteolytic activity was found in their extracts. Several microorganisms isolated on selective media containing yeast cell walls have been found to produce enzymes digesting the yeast wall structures.[5,6] The enzymes used for degrading the walls of Chlorella, Neurospora, and yeasts have not resulted in complete digestion. The most effective enzyme so far reported is that prepared from the bacterium isolated by Masschelein.[6] He observed a decrease of 84% in the turbidity of isolated yeast walls incubated with the enzyme preparations.[6] Nor has the nature of the products released by enzymic degradation of these microbial walls been determined in any detail. Northcote, Goulding, and Horne [1] reported the release of 70% of the total α-cellulose and 43% of the lipid of the wall of *Chlorella pyrenoidosa*. A mannan-protein complex was released from yeast walls treated with papain.[3] Thus at present there is no indication of the nature of the linkages attacked by the various enzymes used in degrading the walls of these microorganisms.

Bacteria. Lysis of bacterial cells and breakdown of the wall has received a great deal of attention, and a variety of enzyme systems is available for various bacterial species.[7–10] Of all the wall-degrading enzymes so far investigated, more is now known about the mode of action of egg-white lysozyme than any other system.[11] The only other enzymes obtained in a purified form and well characterized are the

Streptomyces enzymes studied by Ghuysen.[9] One of these enzymes (Streptomyces F_1) is essentially an N-acetyl-hexosaminidase [12] and is therefore similar to egg-white lysozyme; another enzyme (Streptomyces F_{2B}), an amidase,[13] liberates the peptide moiety from low molecular weight mucopeptides obtained from walls by lysozyme action.[14]

Lysozyme. Although it has been known that lysozyme action on the isolated soluble substrates (usually obtained by chemical fractionation of whole cells) involved the rupture of glycosidic bonds with a liberation of N-acetylamino sugar compounds,[15,16,17] direct evidence establishing the nature of the linkages broken has become available only in recent years.[12,18] The investigation of the nature of the action of lysozyme became simplified when isolated cell walls could be used as "substrate." [19] Using the isolated cell walls of several sensitive organisms, Salton [20] investigated the nature of the products formed on digestion with lysozyme. A complex mixture of fragments resulted, and these fragments were separated into the larger, nondialyzable compounds of about 10,000 to 20,000 molecular weight. These compounds possessed terminal groups of N-acetylamino sugars and contained all of the constituents present in the original wall (but probably in different proportions [20]). About half the original wall of *Micrococcus lysodeikticus* became diffusible upon dissolution with lysozyme. The nature of the diffusible products was investigated, and the most conspicuous "small fragment" detectable was a compound containing glucosamine and muramic acid, probably in the form of a disaccharide.[20] This substance was detected in digests of all three cell walls studied, those of *Bacillus megaterium, Micrococcus lysodeikticus,* and *Sarcina lutea.* Evidence suggested that both amino groups of the amino sugars were acetylated and that the disaccharide possessed a free carboxyl group—that of muramic acid. It

was suggested that the "disaccharide" formed an important structural unit of the cell-wall mucocomplex.[20] Additional products reacting more weakly with various spray reagents were detectable in the dialyzable fractions, but their nature remained unknown until their recent isolation and characterization.[14, 21]

In our investigation in 1959 we were able to confirm the nature of the disaccharide and suggest the structure of this compound. The isolated disaccharides from *Micrococcus lysodeikticus* walls digested with egg-white lysozyme and Streptomyces F_1 enzyme were investigated by reaction of the compounds with $NaBH_4$ and by degradation with β-glucosidase. The products of reaction of the compounds with $NaBH_4$ clearly established the identity of the reducing group liberated by lysozyme action as that of muramic acid, thus providing direct experimental evidence for the hypothetical structure of the lysozyme substrate proposed by Brumfitt, Wardlaw, and Park.[22] The breakdown of the disaccharides into the free N-acetylamino sugars, N-acetylglucosamine and N-acetylmuramic acid, provided evidence of the β-glycosidic bond. The structure of the disaccharide and the nature of the products formed from reaction with $NaBH_4$ and β-glucosidase are presented in Fig. 12. The presence of a $1 \rightarrow 6$ linkage was suggested from experiments performed on [^{14}C] disaccharide oxidized with $NaIO_4$ and determining the recovery of [^{14}C] formaldehyde.[12, 23]

In addition to the disaccharide, an oligosaccharide yielding glucosamine and muramic acid on hydrolysis was detected, and its structure investigated, by the techniques used in studying the disaccharide. That the compound was a tetrasaccharide was supported by measuring the ratios of glucosamine, muramic acid, and "muramicitol" (the amino sugar hexitol of muramic acid [12, 23]) separated after hydrolysis of the substance reduced with $NaBH_4$. Both lysozyme

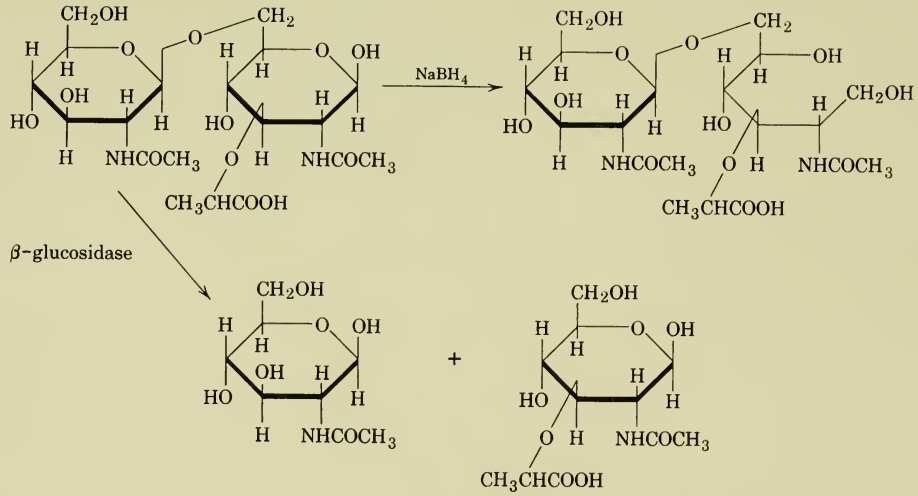


Fig. 12. 6-O-β-N-acetylglucosaminyl-N-acetylmuramic acid and its degradation with $NaBH_4$ and β-glucosidase.[12, 23]

and Streptomyces F_1 enzyme yielded disaccharide from the tetrasaccharide, although the activity of the latter enzyme was much weaker than that of lysozyme.[13] The two enzymes, moreover, are capable of degrading mono- and di-chitibiose (i.e. the di- and tetrasaccharides of N-acetyl-glucosamine), thus clearly showing that they possess $\beta(1 \rightarrow 4)$ N-acetylglucosaminidase activity.[12, 23] This also confirmed the earlier conclusion by Berger and Weiser [18] that the limited action of lysozyme on purified chitin indicated its β-glucosaminidase properties. The experimental evidence is therefore in accordance with the structure of the tetrasaccharide being a $\beta(1 \rightarrow 4)$ dimer of the disaccharide, as shown in Fig. 13.

Independent confirmation that the disaccharide is the simplest product of lysozyme action on its substrate in the cell-wall mucopeptide of *Micrococcus lysodeikticus* has come from the investigations of Perkins,[24, 25] and the structure suggested is identical to that proposed by Salton and Ghuysen.[12] A disaccharide of N-acetylglucosamine and N-acetylmuramic acid has also been detected in partial acid hydrolysates of walls of *Micrococcus lysodeikticus*.[21, 26]

The nature of the fragments obtained on digestion of walls with lysozyme has provided us with an idea of the structure of the cell-wall mucopeptide. Thus the wall

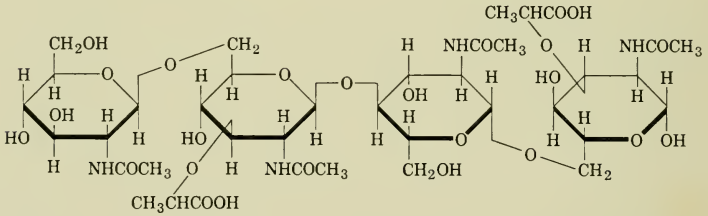


Fig. 13. Proposed structure of the tetrasaccharide enzymically released from *Micrococcus lysodeikticus* walls.[12, 23]

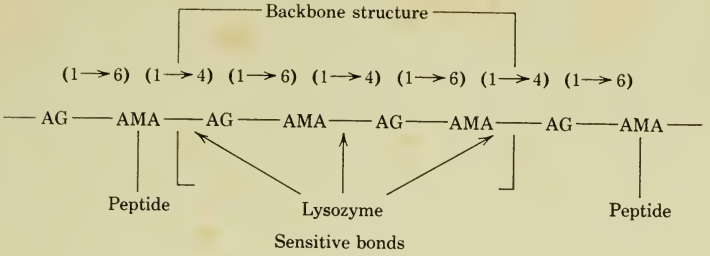


Fig. 14. Backbone structure proposed for *Micrococcus lysodeikticus* wall.[23]

probably possesses a backbone structure of alternating groups of N-acetylmuramic acid and N-acetylglucosamine with alternating $\beta(1 \rightarrow 4)$ and $\beta(1 \rightarrow 6)$ linkages. Some of the muramic acid residues would have peptide substituents, and the possible structure of the wall and the distribution of lysozyme sensitive bonds is shown in Fig. 14.

The isolation of a small molecular weight mucopeptide in the dialyzable fraction of lysozyme-digested walls of *Micrococcus lysodeikticus* [14] has clearly shown that lysozyme can degrade the backbone down to a disaccharide residue possessing a peptide linked through muramic acid, as in the structure in Fig. 15, which shows in addition the linkage sensitive to the Streptomyces amidase.[13]

Although it is now possible to understand the manner in which lysozyme degrades the bacterial walls, yielding a variety of products, some containing all of the parent amino acids and amino sugars in the same molar proportions as in the intact cell wall (e.g. the diffusible mucopeptide) as well as the di- and tetrasaccharides, many of the general problems of understanding lysozyme sensitivity remain to be solved. O-acetyl substituents have been shown to be important in governing this sensitivity in mutant strains of

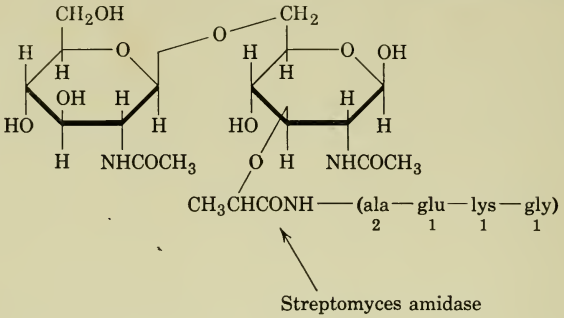


Fig. 15. Structure of mucopeptide in lysozyme digest and bond sensitive to streptomyces amidase.[13]

Micrococcus lysodeikticus,[22] but in other walls O-esters cannot account for the greater resistance of the walls to digestion with lysozyme. The possibility of different linkages between amino sugars of the backbone has been suggested.[27] Resistance to lysozyme could also be explained by differences in the ratios of amino sugars, relatively few disaccharide units, branching points, single amino acid substituents attached to muramic acid, and a high frequency of cross-linked peptides between muramic acid residues (two types of structures discussed in Chapter 2). There are many intriguing possibilities, and it will be of great interest to find out the factors responsible for the resistance of the walls of an organism such as *Bacillus cereus,* which contains such a large amount of amino sugar in the wall (30%).[27]

Biosynthesis of Microbial Walls

The biosynthesis of microbial walls is now beginning to attract much attention, and within the brief space of the last couple of years a great deal has been learned. The discovery of the accumulation of uridine nucleotides in

penicillin-treated *Staphylococcus aureus* by Park and Johnson [28] and Park [29] and the subsequent recognition of the biochemical significance of these compounds by Park and Strominger [30] stimulated a great deal of interest in the mode of action of penicillin and the mechanism of biosynthesis of bacterial cell walls. Much of the work on wall biosynthesis has thus been confined to recognizing wall intermediates accumulating in the presence of various antibiotic inhibitors and has been performed mainly with bacterial cells.

Yeasts and Fungi. So far as I am aware, there have been no direct studies of the biosynthesis of walls of yeasts or fungi. However, it is well known that possible intermediates in the form of nucleotide anhydrides occur in yeasts. Uridine diphospho-(UDP)-glucose,[31] UDP-acetylglucosamine,[32] and guanosine diphospho-(GDP)-mannose [33] could all be regarded as potential wall intermediates, since the monosaccharide moieties of all three nucleotides occur in the walls of yeasts. Although there have been no direct observations involving a transfer of the sugar moieties of these nucleotides into wall compounds, it is conceivable that they may well follow the known transglycosylation reactions [34,35] established for uridine nucleotides and following the general type of reaction given below:

$$\text{UDP-X} + \text{ROH} \rightleftharpoons \text{UDP} + \text{RO-X}$$

Glaser and Brown [36] have investigated the biosynthesis of chitin by extracts of *Neurospora crassa,* which is known to contain poly-N-acetylglucosamine in the mycelia.[37] Enzyme preparations catalyzed the synthesis of chitin by the following reaction:

$$\text{UDP-acetylglucosamine} + (\text{chitodextrin})_n \rightleftharpoons \text{UDP} + (\text{chitodextrin})_{n+1}$$

Chitin in an insoluble form was thus synthesized from UDP-acetylglucosamine, soluble chitodextrin, and an activator.[36] Higher molecular weight chitodextrin was the most effective primer. The enzyme system was in a particulate form.

Bacterial Cell Walls. Three main lines of investigation have been pursued in studies of the biosynthesis of bacterial walls. They include (1) biosynthesis of individual wall compounds, (2) synthesis of mucopeptide and incorporation of radioactive compounds into walls, and (3) isolation and characterization of possible intermediates accumulating during inhibition of wall synthesis.

Biosynthesis of Muramic Acid. Attempts to elucidate the origin of the O-lactyl side chain of muramic acid were made by Strominger.[38] He discovered that *Staphylococcus aureus* contained an enzyme catalyzing the transfer of pyruvate from 2-phosphoenolpyruvate (PEP) to UDP-acetylglucosamine by the following reaction:

$$\text{UDP-AG} + \text{PEP} \rightarrow \text{UDP-AG-pyruvate} + \text{Pi}$$

Although the rate of the reaction in this enzyme system was about one fifth of the rate of UDP-acetylglucosamine formation, Strominger [38] has suggested that in 10 minutes at 37° the enzyme could synthesize sufficient substituted N-acetylglucosamine required for the wall of *Staphylococcus aureus,* thus achieving this feat well within the mean generation time of the organism.

Strominger and Scott [39] have also detected a small enzymic conversion of UDP-acetylglucosamine-[^{14}C]-pyruvate to UDP-acetylglucosamine-[^{14}C]-lactic acid by extracts of *Staphylococcus aureus.* The net reaction, however, was small, and the mechanism of synthesis of muramic acid and its uridine nucleotides still remains to be established.

That the 3-O-carboxyethyl residue of muramic acid is derived from pyruvate was further substantiated in recent experiments performed by Richmond and Perkins [40] with intact cells of *Staphylococcus aureus,* incubated under conditions favoring only cell-wall synthesis.[41] A cell wall synthesized with [^{14}C] glucose in the absence of alanine showed similar specific activities per microgram of carbon for both glucosamine and muramic acid. However, when the wall was synthesized in the presence of alanine, the muramic acid formed had the specific activity of the side chain reduced by 75%. Generally labeled [^{14}C] alanine and [^{14}C] aspartic acid could act as precursors of the muramic acid side chain. The two noncarboxyl atoms of the side chain of muramic acid yield acetaldehyde when heated at 100° with 86% sulfuric acid for 17 minutes (Strange and Kent [42]), whereas the carboxyl group yields carbon monoxide. These facts enabled Richmond and Perkins [40] to conclude from experiments with [^{14}C] alanine that all three carbon atoms of the alanine can act as precursors of the muramic acid side chain without inversion and that these results are consistent with the idea that phosphoenolpyruvate is the immediate precursor.

Synthesis of Wall Mucopeptides and Incorporation of Radioactive Compounds into Walls. The synthesis of cell-wall mucopeptides by washed suspensions of two different strains of *Staphylococcus aureus* in defined incubation mixtures was independently reported by Mandelstam and Rogers [41, 43] and by Hancock and Park.[44] The increase in wall mucopeptide content of *Staphylococcus aureus* incubated in buffers containing glucose and various combinations of the amino acids known to occur in the wall varied from 20 to 150% in one hour at 37°. The results of the experiments performed by Mandelstam and Rogers [41] are pre-

sented in Table 21. Little mucopeptide synthesis occurred in the presence of glucose alone, but a net increase of about 60% took place when glycine or ammonium chloride was added to the glucose. Thus Mandelstam and Rogers [41] showed that it was possible to study the synthesis of wall mucopeptides dissociated to a large measure from protein synthesis.

Hancock and Park [44] studied the incorporation of [^{14}C] amino acids into cell-wall and protein fractions of *Staphylococcus aureus* in the presence and absence of chloramphenicol. They showed that the incorporation of typical cell-wall amino acids, such as lysine, glycine, alanine, and glutamic acid, into the wall was inhibited only to the extent of 4 to 8% by chloramphenicol when the cells were transferred

TABLE 21

Conditions for the Synthesis of Mucopeptide

Washed staphylococci incubated 1 hour in buffer containing 1% glucose and one or more amino acids at a final concentration of 400 μg/ml. Bacteria disintegrated and mucopeptide isolated.

Additions	Increase in Mucopeptide %
None	0–10
DL-Lysine	20
DL-Glutamic acid	25
DL-Alanine	20
Glycine	55–80
Glycine + DL-glutamic acid	60
Glycine + DL-lysine	80
Glycine + DL-lysine + DL-glutamic acid + DL alanine	100–150

Reference 41.

to a synthetic growth medium containing the radioactive amino acids. On the other hand, the inhibition of the incorporation into protein of the "wall" amino acids and leucine, proline, and phenylalanine was as much as 85 to 98%. In agreement with the investigations reported by Mandelstam and Rogers,[43] Hancock and Park [44] were also able to demonstrate a doubling of the amount of wall (measured by incorporation of [^{14}C] lysine and glycine) in a simple incubation mixture containing lysine, glycine, alanine, glutamic acid, glucose, and uracil but lacking in some of the amino acids essential for protein synthesis. Under these conditions chloramphenicol had no effect on wall synthesis. An examination of the wall formed in the presence of chloramphenicol suggested that it was normal in that the ratios of increase in glutamic acid, glycine, alanine, lysine, and hexosamine (1:5.8:4.2:2.0:1.8) were similar to those present in the initial wall (1:6.3:2.8:1.9:1.8).

The synthesis of the cell-wall mucopeptide was markedly inhibited by penicillin and bacitracin, neither of which inhibited protein synthesis.[41] However, a small amount of mucopeptide is synthesized in the presence of penicillin, and Mandelstam and Rogers [41] found some evidence suggesting that it possessed an abnormal composition. Nathenson and Strominger [45] also studied the inhibitory effect of penicillin on the incorporation of [^{14}C] lysine and [^{32}P] into wall and cellular protein and nucleic acid of *Staphylococcus aureus* and the incorporation of [^{3}H] diaminopimelic acid and [^{14}C] glucose into the wall of *Escherichia coli*. The results presented in Table 22 are again in accord with those of other investigators, showing a marked inhibition of amino acid incorporation into the cell wall but allowing both protein and nucleic acid synthesis to proceed in the presence of penicillin. The inhibition by penicillin of [^{14}C] glucose incorporation into the whole cell wall of *Escherichia*

TABLE 22

Effects of Penicillin on Incorporation of Isotopes into Cell Wall or into Cell Protein and Nucleic Acid in *Staphylococcus aureus* and in *Escherichia coli*

	Staphyloccus aureus				*Escherichia coli*	
	C^{14}-Lysine		P^{32}-Inorganic phosphate		H^3-DAP	C^{14}-Glucose
Isotope	Cell-Wall	Protein	Cell-Wall	Nucleic Acid	Cell-Wall	Cell-Wall
Control	34,800	5,100	155,000	11,600	1,040,000	389,000
+ Penicillin	3,290	4,960	48,900	11,600	297,000	334,000
% Inhibition	91%	2%	68%	0	72%	14%

Data are expressed as specific activities (cpm/mg).
Reference 45.

coli is not marked, and indeed Trucco and Pardee [46] in earlier experiments had concluded that penicillin did not interfere with the synthesis of the wall of this organism. As *Escherichia coli* wall possesses major protein, lipid, and polysaccharide components, it seems likely that inhibition of incorporation of the compounds into the mucopeptide fraction of the wall of this Gram-negative organism may have been more spectacular. Inhibition of mucopeptide synthesis by penicillin has been clearly established by at least three groups of investigators for *Staphylococcus aureus*. The results of interference with wall synthesis in *Escherichia coli* are conflicting, and Meadow [47] has recently found that a DAP-requiring mutant of *Escherichia coli* is unchanged in its ability to incorporate [^{14}C] glucose [^{14}C] lysine and [^{14}C] diaminopimelic acid into wall during the first 30

minutes of exposure to penicillin. To what extent this lack of agreement is a reflection of strain differences has not been determined.

It is of interest to note that the partially disrupted cells of *Staphylococcus aureus* investigated by Gale and Folkes [48] will incorporate a high proportion of the total [^{14}C] amino acid uptake of an amino acid such as glycine into the trichloroacetic acid insoluble, cell-wall fraction (Gale, Shepherd, and Folkes [49]). Whether the mucopeptide was in the form of finished wall or simply TCA-precipitable material was not established. It would be of interest to know whether the disrupted cells retain an intimate contact between the acceptor cell wall and the sites of new mucopeptide synthesis. In attempting to localize the sites of synthesis of mucopeptides, Brookes, Crathorn, and Hunter [50] have investigated the time course of the uptake of [^{14}C] amino acids (L-alanine, diaminopimelic acid, and L-aspartic acid) into the wall, membrane, and protoplasmic fractions of *Bacillus megaterium*. They concluded from their results that the mucopeptide components are synthesized at sites on or closely associated with the cytoplasmic membrane.

Incorporation of* [1-^{14}C] α, β-*methyl-N-acetyl-D-glucosaminide into Cell Walls. The organism *Lactobacillus bifidus* var. *pennsylvanicus* has a specific growth requirement for N-acetyl-D-glucosamine, preferably in the form of β-glycosides.[51] That this requirement was associated with wall-synthesizing systems has been shown by Zilliken's experiments [52,53] in which the proportion of morphologically bizarre and bifid forms decreases with increasing amounts of the glucosaminide growth factors; [1-^{14}C] β-methyl-N-acetyl-D-glucosaminide is incorporated into the cell walls of *Lactobacillus bifidus,* the specific activity in the muramic acid being 19,000 cpm/mole compared to 21,000 cpm/mole for the starting material. Zilliken [52,53] concluded that N-acetyl-

D-glucosamine is a direct precursor of muramic acid and that the latter compound is indeed a D-glucosamine derivative.

Incorporation of [^{14}C] Lysine, [^{14}C] Diaminopimelic Acid, and [^{14}C] Glucose into Cell-Wall Lysine and DAP. Meadow and Work [54] investigated the incorporation of radioactive compounds into wall fractions of *Escherichia coli* mutants requiring either lysine or DAP or both amino acids for growth. All of the mutants tested took up [^{14}C] lysine, and of the radioactivity incorporated 50 to 60% was accounted for in the cell walls. DAP was not labeled. Ten per cent of the cell-wall lysine of the DAP-requiring mutants was derived from that supplied exogenously; [^{14}C] diaminopimelic acid was incorporated into both DAP and lysine of the DAP-requiring mutants and the parent strain. The DAP-dependent, lysine-stimulated mutant 173-25 derived 80% of the cell-wall lysine from the exogenous DAP, whereas the corresponding value for the DAP-dependent mutant was 50 to 60%. An alternative route to lysine from glucose was apparent from mutants 173-25 and DAP-dependent grown on [^{14}C] glucose. Labeling of lysine occurred, but DAP was unlabeled. Some 10 to 20% of the cell-wall lysine was derived from glucose in these mutants.

Accumulation and Identification of Cell-Wall Intermediates. The identification of cell-wall intermediates really commenced with the discovery of the accumulation in *Staphylococcus aureus* of uridine nucleotides in the presence of penicillin.[28,29] The significance of these nucleotides as possible cell-wall precursors became apparent when the amino sugar was found to be identical to muramic acid and the complete structure for one of the nucleotides was established, as in Fig. 16.[30] This finding was just preceded by Lederberg's [55,56] suggestion that penicillin acted

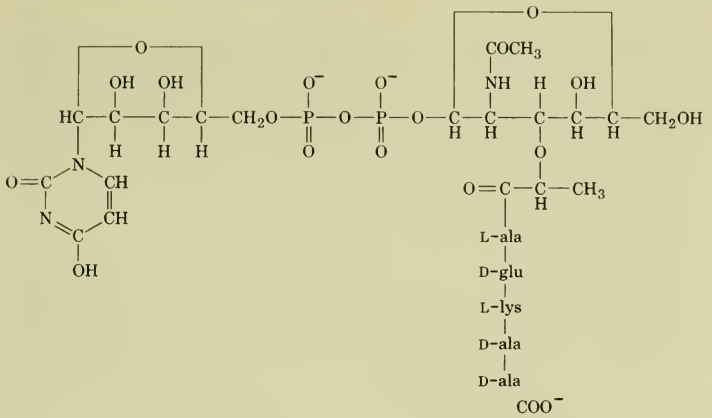
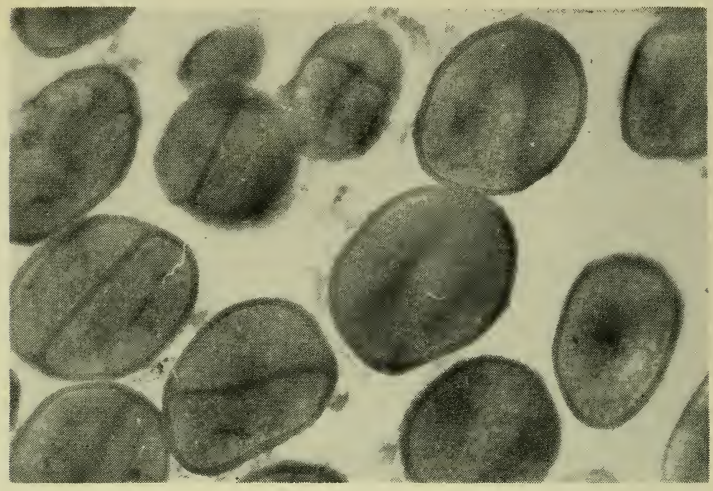
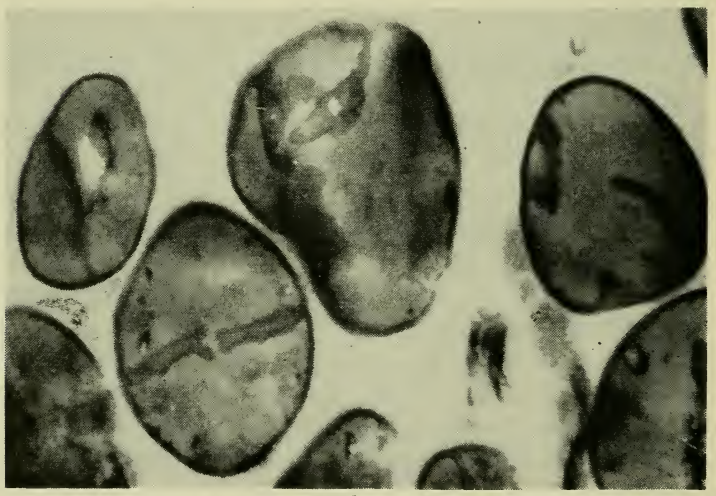


Fig. 16. Structure of the uridine nucleotide from penicillin-inhibited *Staphylococcus aureus*.[30]

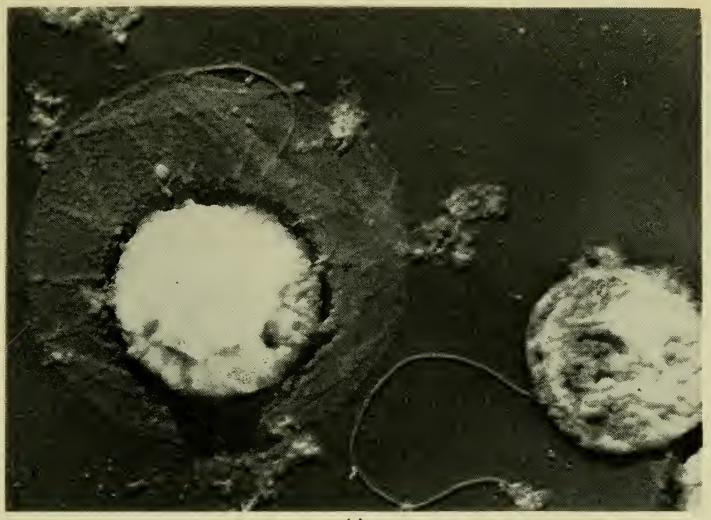
on bacteria (including the Gram-negative *Escherichia coli*) by blocking wall formation. Thus the biochemical and structural evidence for the inhibition of wall formation by penicillin emerged and has been largely confirmed in many subsequent studies. The anatomical lesion caused by penicillin inhibition of wall formation is beautifully illustrated in the thin sections of *Staphylococcus aureus* shown in Fig. 17*a* and *b,* taken from the studies of Murray, Francombe, and Mayall.[57] The consequences of inhibition of the formation of the mucopeptide part of the wall of Gram-negative bacteria has become apparent from a number of investigations.[58–60] However, the Gram-negative bacteria have major wall constituents unaffected by penicillin action, and the familiar "poached-egg" appearance of "protoplasts" of *Vibrio metchnikovi* formed in the presence of penicillin is shown in Fig. 17*c*.



(*a*)



(*b*)



(*c*)

Fig. 17. Effects of penicillin on cell-wall structure. (*a*) Thin section of untreated cells of *Staphylococcus aureus* (×36,000); (*b*) effects of exposing *Staphylococcus aureus* to penicillin for three hours (×41,500). From the study of Murray, Francombe, and Mayall (Ref. 57). (*c*) *Vibrio metchnikovi* "protoplasts" prepared by growth in the presence of penicillin. The weakened wall from the right-hand "protoplast" became detached during preparation for electron microscopy (×12,500). M. R. J. Salton, unpublished.

Since the early investigations of Park and Johnson [28] and Park,[29] a number of uridine nucleotides containing typical wall components has been isolated from untreated cells as well as from organisms whose growth has been inhibited by antibiotics or deprivation of specific amino acids. Baddiley et al.[61] isolated cytidine diphosphoribitol and cytidine di-

phosphoglycerol from normal cells of *Lactobacillus arabinosus,* and the search for the biochemical functions of these nucleotides led them to the discovery of the cell-wall teichoic acids. Cell-wall nucleotide intermediates have been found in both Gram-positive and Gram-negative bacteria, and some of the nucleotides so far identified are listed in Table 23.

Nucleotide accumulation also occurs with the antibiotics bacitracin [71] and novobiocin,[65] and recent studies by two groups of workers have shown that 5-fluorouracil induces nucleotides to accumulate.[72,73] The precise manner in which penicillin brings about the accumulation of the vari-

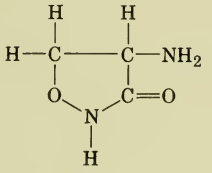
TABLE 23

Nucleotides Identified as Probable Cell-Wall Intermediates in Various Bacteria

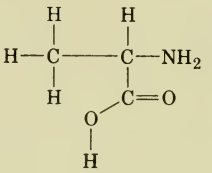
Organism	Inhibitor	Nucleotide
Staphylococcus aureus	Penicillin	UDP-AG-lact-ala-glu-lys-ala-ala
		UDP-AG-lact-ala
		UDP-AG-lact *
	Oxamycin	UDP-AG-lact-ala-glu-lys
	Lysine-deprivation	UDP-AG-lact-ala-glu
		UDP-AG-lact-ala
	Gentian violet	CDP-ribitol
Escherichia coli (DAP-dependent mutant)	None	UDP-AG-lact-ala-glu-DAP-ala-ala
	DAP-deprivation	UDP-AG-lact-ala-glu
Streptococcus (Group A)	None	UDP-AG-lact
Lactobacillus arabinosus	None	CDP-ribitol

* UDP-AG-lact = uridine diphospho-N-acetylmuramic acid.
References 28–30, 35, 61–70.

ous nucleotides is not known. At least the probable mechanism of the antibiotic action of oxamycin (D-cycloserine) has been amenable to study almost at the level of a single enzyme system. Direct evidence for the inhibition of wall synthesis came from the studies of Shockman [74] using *Streptococcus faecalis* and from Strominger, Threnn, and Scott [63] with *Staphylococcus aureus*. Both investigations led to the conclusion that oxamycin was acting as a competitive antagonist of the incorporation of D-alanine into wall. Strominger [35] has pointed out the close structural relationship of oxamycin to D-alanine as shown below:



Oxamycin



D-alanine

Nucleotides isolated from oxamycin-inhibited cells have given some further evidence of the sequence of the building up of the wall peptide, and Strominger, Threnn, and Scott [63] have shown (Table 24) that the nucleotide accumulation induced by oxamycin can be antagonized by D-alanine. Strominger [35] has thus suggested that oxamycin inhibits the enzymic reaction involved in the addition of D-alanine to the nucleotide UDP-AG-lact-ala-glu-lys.

It is curious that none of the cell-wall intermediates so far isolated from *Staphylococcus aureus* contains either glycine or N-acetylglucosamine, the other two major cell-wall constituents. It may well be that the peptides attached to the nucleotides isolated up to the present time are far from complete, despite the remarkable similarity in their amino

TABLE 24

Antagonism by D-alanine of Uridine Nucleotide Accumulation Induced by Oxamycin

Antagonist Added	Experiment 1	Experiment 2
None	41.4	30.0
D-alanine (500 μg/ml)	17.0	12.1
D-alanine (5000 μg/ml)	4.5	6.9
L-alanine (5000 μg/ml)	41.5	32.2
DL-alanyl-DL-alanine (5000 μg/ml)		33.5
D-serine (5000 μg/ml)		34.2

In *Experiment 1* oxamycin (75 μg/ml) and possible antagonists were added together at 0 time. In *Experiment 2* oxamycin (75 μg/ml) was added at 0 time. At 45 minutes, 20.4 μM of nucleotide had accumulated. At this time possible antagonists were added and incubation was continued for 45 minutes longer. Data are expressed as μmoles of uridine nucleotide per liter of culture at half-maximal growth.

Reference 63.

acid composition to that of the cell-wall mucopeptide [75] and even very close agreement in the proportions of the D- and L-alanine,[65] as shown in Table 25. Of course, an alternative explanation of the presence of glycine in a "special" peptide or structure would equally well explain its absence from the nucleotides.

Enzymic Synthesis of Wall-Precursor Nucleotides. Conditions leading to the formation of a uridine nucleotide have been investigated for only one of the cell-wall intermediates. Ito and Strominger [76] have found that an enzyme from *Staphylococcus aureus* purified about 500-fold, will catalyze the formation of UDP-AG-lact-ala-glu-lys under the conditions summarized in Table 26.

TABLE 25

Optical Configuration of Alanine Samples Obtained from a Uridine Nucleotide and from the Cell Wall of *Staphylococcus aureus*

Alanine Samples	% L-alanine	% D-alanine
1. UDP-AG-lact-peptide from novobiocin-inhibited *Staphylococcus aureus* (strain Copenhagen)	32.1	68.0
2. UDP-AG-lact-peptide from penicillin-inhibited *Staphylococcus aureus* (strain H)	33.7	65.6
3. Cell wall, prep. 1	27.3	65.4
4. Cell wall, prep. 2	33.4	66.2
5. Cell wall, prep. 3	34.0	65.7

Reference 65.

TABLE 26

Requirements for the Enzymatic Synthesis of UDP-GNAc-Lactyl-(L)Ala-(D)Glu-(L)Lys

Experiment 1: System	Cpm	Experiment 2: Nucleotide Added	Cpm
Complete	2620	UDP-GNAc-lactyl-ala-glu	5080
-UDP-GNAc-lactyl-ala-glu	0	UDP-GNAc-lactyl-ala	0
-ATP	0	UDP-GNAc-lactyl-ala-glu-lys	6
-Mg++	0	UDP-GNAc-lactyl-ala-glu-lys ala-ala	0

In *Experiment 1* various components were omitted from the complete incubation mixture, which contained buffer, $MgCl_2$, C^{14}-lysine, ATP, UDP-GNAc-lactyl-(L)ala-(D)glu, and purified enzyme. In *Experiment 2* other uridine nucleotides were substituted for UDP-GNAc-lactyl-(L)ala-(D)glu. Data are expressed as cpm of C^{14}-lysine incorporated into nucleotide, measured as charcoal-adsorbable radioactivity.

Reference 76.

Pathways for Cell-Wall Biosynthesis

The evidence that the uridine and cytidine nucleotides containing a number of typical cell-wall compounds are indeed cell-wall intermediates is most convincing when their compositions are compared with cell walls. Yet the hard fact remains that convincing incorporation or transfer of the muramic-acid–peptide moiety of the nucleotide to the wall has not been demonstrated. This, of course, has prompted the sceptics to say "I told you so!" However, the problem of getting such a nucleotide through the existing wall into the right part of the membrane and close to acceptor sites on the wall must be a tremendous one. Strominger has also clearly pointed out that very low levels of transfer would not be surprising if one attempted to assess the probable number of acceptor sites on the wall. "If it is assumed that intact organisms contain of the order of 1000 acceptor sites per cell, then all the cell walls obtained from a liter of culture containing 10^9 cell per milliliter would contain only 10^{15} acceptor sites or 0.01 μM of acceptor per liter of culture." [35] This problem of experimental demonstration of the transfer of the obvious intermediates into wall is a difficult one and indeed seems to be general to the whole problem of the synthesis of large polymers including cellulose.[77] However, some ideas of the mechanisms of wall biosynthesis are emerging from pioneer work of Strominger and his colleagues.

The biochemical unity of life so admirably discussed by the late Kluyver and Van Niel in their Prather Lectures [78] prompts me to be optimistic and believe that some of the pathways for wall synthesis recently suggested by Strominger [35] will become established as general mechanisms for the synthesis of these most interesting heteropolymers. These schemes, as Park [69] has also pointed out, involve a transfer

mechanism of transglycosylation, commonly encountered in the biosynthesis of many compounds, including the structural polymer chitin. Thus the basis of these reactions involving uridine and cytidine nucleotides (the only two classes so far implicated in bacterial wall synthesis) would be analogous to that found for chitin synthesis by Glaser and Brown.[36]

Nucleotide – wall component + acceptor (wall)

→ acceptor – wall component + nucleotide diphosphate

Two major pathways for biosynthesis of part of the bacterial wall have been suggested by Strominger [35,65] and are presented in Figs. 18 and 19. For those organisms possessing an amino sugar backbone, a further part of the biosynthetic scheme can be suggested, since some of the possible intermediates are already known (UDP-AG-lact, UDP-AG, muramic acid-6 phosphate [79]). This hypothetical pathway illustrated in Fig. 20 could be envisaged as being integrated with the other pathways (Figs. 18, 19), thereby adding the muramic acid–peptide residue to an amino sugar backbone already built on to the cell-wall acceptor.

Epideictic

In the period of the last ten years a new class of structural heteropolymers has been discovered in bacterial cell walls and in at least some of the related blue-green algae. We are just beginning to understand some of the properties and structures of these mucopeptide and mucopolysaccharide substances, and some exciting details of the biosynthesis of the major structural component of microbial cells are beginning to emerge. It is perhaps fortunate for mankind that nature saw fit to encase bacteria in a wall containing amino sugar and amino acid structures not normally en-

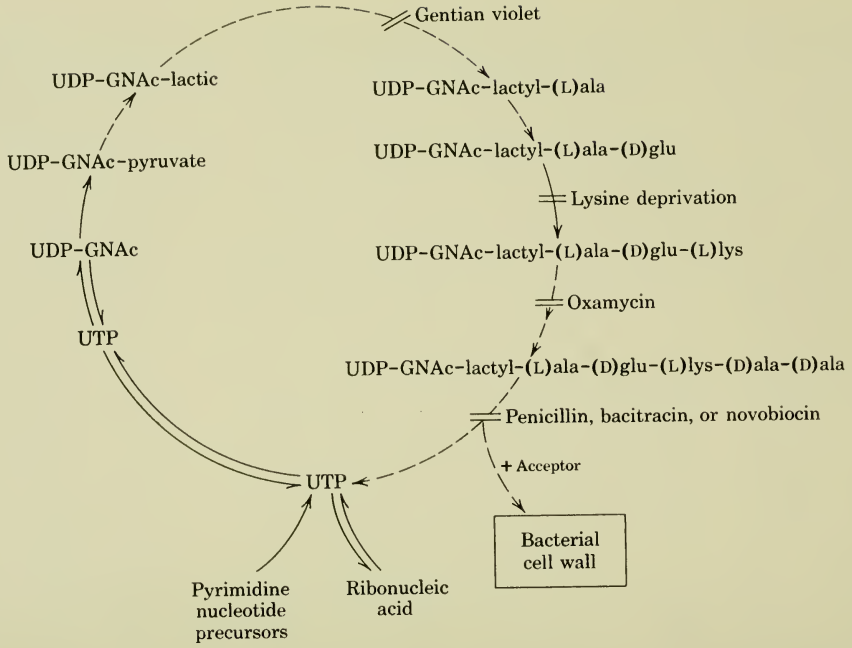


Fig. 18. A scheme for the biosynthesis of part of the bacterial cell wall as suggested by Strominger and Threnn[66] from studies with *Staphylococcus aureus*.

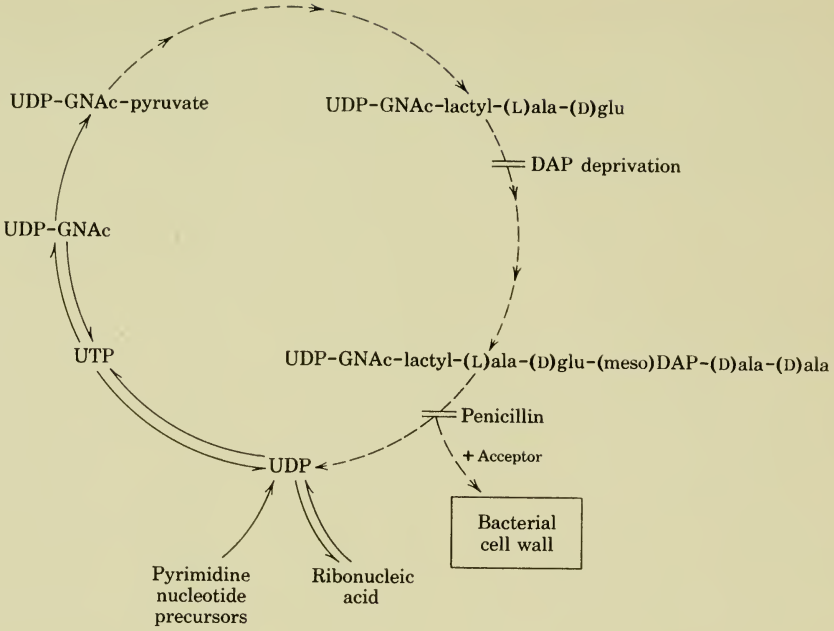


Fig. 19. A scheme for the biosynthesis of part of the cell wall of *Escherichia coli* from the studies of Strominger.[35]

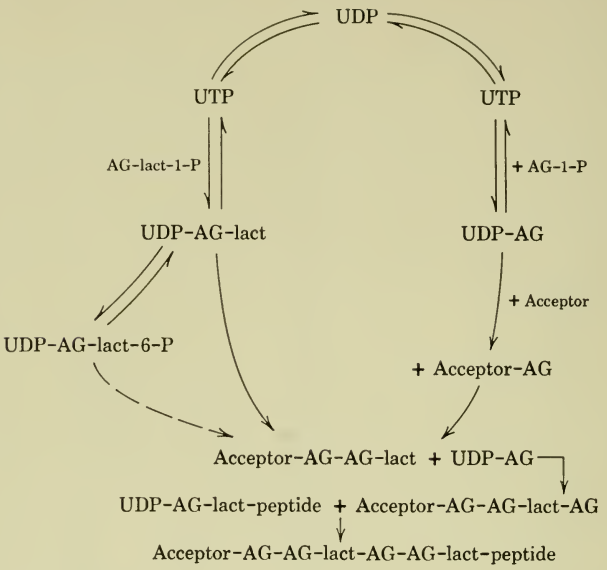


Fig. 20. A hypothetical pathway for the biosynthesis of bacterial cell wall.

countered in higher organisms, for this undoubtedly accounted for the great selective toxicity of the antibiotic penicillin and probably some of the subsequent antibacterial agents.

There is little doubt that the next ten years will see an enormous widening of our understanding of both the chemical structure and biosynthesis of these fascinating structures. If, in the process of unraveling these details, we can add a few strokes to our picture of the evolution of microbial structures and the interrelationships of microbial groups, our scientific curiosity will have been amply rewarded.

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